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REMARKS

1. GENERAL MATTERS

On December 23, 2003, Applicants filed a request to vacate the instant August 26 action on the ground that it failed to consider the papers filed on August 7, 2003. These papers are relevant to one or more of the maintained rejections.

The claim listing assumes entry of the August 6 papers.

2. NEW REJECTIONS

The Examiner has withdrawn the indicated allowability of claims 18-22, 24-41, 51-53, 59, 61, 63, 65-79, and 80-82. (OA §16).

2.1. Description Issues (OA §3).

2.1.1. "corresponds essentially to" (claims 18 and 76) (OA §3a).

The criticized passage was added to claim 18 by the October 2, 2000 amendment, at which time the claimed polypeptide was permitted to be up to 100 amino acids in length. Since the polypeptide is now limited to a length of 20 a.a., this limitation appears unnecessary, and consequently the entire "where" clause has been deleted.

2.1.2. "stabilized" (claims 18-21, 34, 76) (OA §3b).

The Examiner says that the specification describes only one way of stabilizing the peptide, and that is "attaching the small compound with the formula disclosed on the bottom of page 21" to the N-terminal of the claimed peptide.

Stabilization was in fact generically contemplated. Thus, original claim 1 recited "the peptide is stabilized" without any limiting reference to the small compound.

P21, L21-23 teaches that "the synthesis of mimetics can provide compounds exhibiting greater...stability". The specification then continues at P21, L24-P22, L9:

> As an example, the following peptidomimetic has been derived on the basis of the α-helical templates in C-terminal peptide mimetics of cytokines disclosed in US 5,446,128 (97) in combination with the knowledge that the C-terminal of IL-10 exists as an α-helix (98).
>
> [formula omitted]
>
> By this attachment of a small molecule at the N-terminal of the peptide, an α-helical structure of the synthetic peptide is stabilized, and the peptide is made more resistant to proteolytic degradation. Other peptidomimetics may be derived on the basis of the disclosure in US 5,446,128. Such substances wherein substitution has taken place at other residues than X1 and/or substitution has taken place with other molecules than the N-terminal molecule shown in the formula above are within the scope of the present invention.

The compound referred to by the examiner comprises one of the aforementioned alpha-helical templates, and we believe that P21, L24-28 is fairly read as teaching, not just the depicted stabilizing moiety, but also use of any of the alpha-helical templates disclosed in the cited USP 5,446,128. Indeed, P22, L4-5 specifically states as much. This is sufficient to refute the Examiner's contention that only a single means of stabilization is disclosed.

Stabilization is discussed elsewhere in the specification as well. P17, L25-34 teaches that non-natural or unusual amino acids can be introduced into the peptide, and P19, L3-6 suggests that these "alternate [sic] residues can be used (a) to replace chemically reactive residues and improve the stability of the synthetic peptide towards e.g. enzymatic and proteolytic degradation".

Cyclization is also taught by the specification, and it

would be apparent to one skilled in the art that this would stabilize the peptide against degradation by an exopeptidase.

2.1.3. "peptidomimetic" (claim 40) (OA §3c).

The Examiner says that the only adequately disclosed mimetic is the one disclosed on p. 21. As already explained in §2.1.2 of this response, Applicants taught all of the alpha helical templates disclosed in the '128 patent, not just the exemplary one depicted at the bottom of p. 21. Moreover, Applicants may fairly be said to have disclosed the use of alpha-helical mimetics in general. Claim 40 has been amended accordingly.

2.1.4. molecular weights in claim 73 (OA §3d).

Office action 3d is specific to claim 73, which recites that each amino acid has a molecular weight which does not exceed that of "Fmcc-His (Trt)-Opfp (785.78 daltons)". This claim was responsive to criticism that the non-natural AAs were open ended. In the amendment of July 11, 2001, at page 13, we said

> It seems to us that if a limitation is to be imposed, the most logical one would be in terms of molecular weight. This would implicitly limit the number of atoms/complexity of the derivative. **In the Neosystem catalogue, the highest MW amino acid is Fmoc-L-Lys(Trp)-OPFp, MW 745.6, on page 141 (catalogue no. FA01218).** In the Bachem catalogue, it is, not counting certain dimers, Fmoc-His(Trt)-OPfp, MW 785.78, on page 782, catalogue no. B-1650. In the Novochem catalogue, it is L-Thyroxine, MW 776.9, catalogue number 04-11-0011. Therefore, if the nature of the amino acid must be explicitly limited, we would suggest consideration of the limitation, "where each of said remaining amino acids has a molecular weight which is not greater than that of Fmoc-His(Trt)-OPfp" (see new claim 73).

In view of the examiner's comment, and the quoted passage, we have amended claim 73 to remove the parenthetical molecular weight.

2.1.5. "a method of preventing death due to pancreatitis" (claims 80-81) (OA §3e).

The Examiner first states what appears to be an enablement/utility rejection: "instant specification has not disclosed any subjects that were prevented from dying by administering the composition of claim 41".

Examples 13 and 14 describe the use of the analogue IT9302 in the treatment of pancreatitis. Page 48 states that this therapy resulted "in a reduction of mortality from 60% to 0% after 12 hours".

The Examiner has not established a basis for doubting the assertion that the polypeptides of claim 41, which are modified forms of IT9302, would share the therapeutic efficacy of IT9302, especially given (1) Ex. A attached to the July 11, 2001 amendment and (2) the functional limitations of claim 41.

The Examiner also says that "'prevention' means determining in advance if a patient is susceptible to a specific disorder and Applicants are not enabled for such".

It is certainly desirable, for economic reasons if nothing else, to determine in advance if a patient is susceptible to a particular disorder before administering a preventative. However, it is certainly possible to administer the compositions of claim 41 to all human subjects in order to prevent death due to pancreatitis in those subjects. Hence, we do not agree with the Examiner that it is proper to insist that claims 80-81 include a susceptibility determination step.

That said, we believe that such screening would not require undue experimentation. Claim 80 is directed to the prevention

of death due to pancreatitis. Hence, the observation of one or more of the symptoms of pancreatitis would indicate that the subject is susceptible to death due to pancreatis and would warrant preventative action as disclosed. Note that claim 80 recites prevention of "death due to pancreatitis", not prevention of pancreatitis per se.

The specification discloses that there is a relationship between acute pancreatitis and elevated IL-8 levels, and that IT9302 constrains "IL-8-induced leucocyte invasion in the pancreas". P45, L21-27. Hence, IL-8 could be monitored to identify suitable subjects.

The specification also discloses that leukopenia is a symptom of pancreatitis, see P46, L6-8. Other symptoms include elevated TNF-α, amylase, lipase and tryptase in blood, see P48, L8-15. These symptoms can also be used to identify subjects.

In view of the role of I2-10 in regulating TNFα and IL-8, see P6, L12-17, subjects with depressed IL-10 activity would be especially vulnerable. So IL-10 levels can be used, too.

As for claim 81, which recites prevention of ARDS-like syndrome, one mechanism of death to pancreatitis is the development of ARDS-like syndrome, and thus the patients susceptible to death due to pancreatitis are also susceptible to ARDS-like syndrome, per claim 81, see P48, L20-25.

2.2. Definiteness Issues

2.2.1. Reference Table A" (claims 22 and 76) (OA §4a).

Claims 22 and 76 have been amended to explicitly recite all of the amino acids set forth in Reference Table A on p. 18.

2.2.2. Claim 24 (OA §46).

Claim 24 has been cancelled.

2.2.3. "substantially pure" (OA §4c).

As a matter of law, use of term "substantially" does not render a claim indefinite per se. The standard for the term "substantially pure" is established by (1) the level of purity of murine IT9302 as obtainable from Schafer-N, Levsø Parkallé 42, DK-2100 Copenhagen Ø, Denmark "(P44, L15-16), (2) the level of purity of commercially available human IT9302; (3) the level of purity obtainable by the synthetic methods disclosed at P19, L20-P20, L20; and (4) the level of purity necessary for acceptable for the disclosed purpose, i.e., pharmaceutical acceptability, see, e.g., P23, L5-9.

Further particulars as to purification of IT9302 may be obtained from WO96/01318, cited at P1, L18.

3. MAINTAINED REJECTIONS

The viability of the maintained rejections is questionable in view of the Examiner's failure to consider the August 7 supplemental response. While these rejections should be vacated, in the interest of compact prosecution we respond to them anyway.

3.1. Definiteness Issue (OA §2b).

The Examiner maintains the rejection of claims 49, 51-53, 57, 59 and 61 (the rejection of claim 61 is inferred from page 6, line 9 of the December 17, 2002 office action) because they do not recite a "specific disease".

The claim in question defines the diseases functionally, i.e., by whether they are treatable with the disclosed polypeptide, which is said to have certain properties of potential clinical relevance.

It is well established that a claim may contain a functional limitation. See MPEP 2173.05(g). It can readily be determined whether a given disease qualifies; this simply requires administering a claimed compound, known to be active, to a

subject suffering from the disease and see if the subject responds. One can predict whether a disease is likely to be treatable by considering whether the disease is one where macrophages/T-lymphocyte mediated immune reactions are considered pathogenetically important or where an immune modulator with IL-10-like activity is likely to be therapeutically important, e.g., it is responsive to IL-10 or at least is characterized by excessive levels of the hormones down-regulated by IL-10 or deficiencies in the hormones up-regulated by IL-10. Such a prediction has already been made for the numerous diseases specifically enumerated in Tables 1 and 2.

It would not be equitable to limit Applicants' scope of protection to these known diseases; as other diseases are identified as responsive, it would be logical to apply the instant invention to their resolution.

In the April 9, 2003 paper, we demonstrated that numerous patents have issued in which claims have defined therapeutic methods in terms of "diseases treatable" with a particular agent or "diseases mediated" by a particular biomolecular entity.

Prior U.S. patents may properly be used as evidence that there is a consensus that particular claim language is accepted as definite. Ex parte Brian, 118 USPQ 242, 245 (POBA 1958) cited the past practice of the office in allowing "fingerprint" claims, and in Andrew Corp. v. Gabriel Electronics, Inc., 6 USPQ2d 2010, 2012 (Fed. Cir. 1988), the Court of Appeals said that the term "substantially" was "ubiquitous" in patent claims and therefore definite. See also Vitronics Corp. v. Conceptronic Inc. 39 USPQ2d 1573, 1578-9 (Fed. Cir. 1996); In re Cortright, 49 USPQ2d 1464 (Fed. Cir. 1999).

3.2. Enablement Issues (OA §2a).

3.2.1. Claim 49 The Examiner concedes that the specification is enabling for treatment of arthritis,

pancreatitis and ARDS-like syndrome, but rejects claim 49 insofar as it attempts to embrace treatment of any disease treatable by a substance which has the properties recited in a-k of claim 49.

The Examiner says that to satisfy 35 USC 112 ¶1, the instant specification "must provide guidance as to which diseases might be treatable with said substances".

The claimed compounds are "IL-10 agonists" (P1, L4). There activities, as enumerated in the specification, include antagonism of IL-8, IL-1β, MCAP/MCP-1, IFN-γ and TNF α, and induction of IRAP and IL-4 production (PP. 2-3, 6-7).

Based on this, applicants list over 40 diseases in Table 2 "where an immune modulator with IL-10-like activity, due to its induction of IRAP production and/or inhibition of cytokine production and/or activity may have therapeutic importance", citing refs. 20-74 and 109.

They also discuss, more generally, the role of IL-10 in the cellular immune system and suggest that their IL-10 agonist could be useful in treating over 40 diseases where "macrophages/T-lymphocyte-mediated immune reactions are considered pathogenetically important", see Table 1.

It thus appears that the specification provides substantial guidance as to which diseases might be treatable.

The Examiner adds that there also has to be "some expectation of success that the diseases are treatable with these substances". Applicants have demonstrated utility in connection with arthritis, pancreatitis and ARDS-like syndrome. Arthritis has no general clinical association with pancreatitis or ARDS-like syndrome. This is supportive of an expectation of success with diverse diseases.

The utility in connection with the diseases of Table 2 is based on the known activity of IL-10 and the cytokine profiles of the sufferers. That in connection with the diseases of Table

1 is based on the known associations of the diseases in question with the cellular immune system for dysfunction thereof). These associations provide a reasonable expectation of success.

The general therapeutic applicability of the synthetic analogues is supported by the facts that the IL-10R2 receptor, which binds the C-terminal part of IL-10, is expressed in all cell types except cells in the brain, and that the analogues can block IL-1 inducible effects.

Moreover, as reported in the enclosed declarations, the inventors have found that IL-10 and its synthetic analogues have regulatory effects on NF-κB. NF-κB again has a long list of known target genes as reviewed in the enclosed publication of Pahl in Oncogene (1999) 18, 6853-6866. Among the target genes are cytokines, immunoreceptors, growth factors and enzymes suggesting that IL-10-mediated regulation of NF-κB may have effects on the development of many, diverse types of disorders.

Ex parte Mark, 12 USPQ2d 1904 (BPAI 1989) stands for the proposition that a claim can have an activity limitation so that it does not inadvertently read on inoperative embodiments. The treatability limitation here serves a similar purpose.

The aforementioned U.S. patents act as evidence that determined which diseases are treatable with or mediated by a particular substance is not considered to require undue experimentation. For the propriety of such evidence, see the cases cited in 3.1 above.

3.2.2. The Examiner also rejects claim 87, which recites diseases "characterized by decreased or insufficient production, or decreased or insufficient activity, of IL-10".

The Examiner argues that it would require undue experimentation to determine "all" diseases so characterized. This test is improper as a matter of law. Just as the fact that a compound claim covers thousands of compounds does not mean that

all of the covered compounds must be tested see In re Angstadt, 190 USPQ 214, 218 (CCPA 1976), the fact that a method claim covers hundreds (or even thousands) of diseases does not mean that the relevant quantum of experimentation is that required to test all of the diseases.

Rather, the proper standard is the amount of experimentation necessary to determine whether a randomly chosen disease is included. For claim 87, this would require measurement of IL-10 production and activity, which is quite conventional.

3.2.3. The Examiner rejects claim 57 which recites a method of treating cancer. The Examiner explains that "cancer is not one disease, it is a very complex condition which encompasses various types of malignant neoplasms, and all cancers are not caused by the same culprits or treated by the same agents".

As the Examiner is aware, some cancer treatments, e.g., active specific immunotherapy, target features specific to a particular kind of cancer, and would not be expected to affect highly diverse cancers. Other treatments are more generic in nature, e.g., they target rapidly dividing cells (radiotherapy, chemotherapy) or areas with leaky blood vessels (IR absorption therapy), and hence are applicable to any accessible cancer.

IL-10 is involved in processes that are central in the development of virtually all types of cancer. This is supported by the studies described in the enclosed declarations showing that IL-10 and the synthetic analogues are capable of inhibiting the processes of cell migration, cell proliferation and angiogenesis.

Interesting in this context is the previously established regulation of cyclin D1 transcription by NF-κB as mentioned on page 6859 in the Pahl reference. Cyclin D1 is a well-characterised regulator of cell cycle progression and a protooncogene. Ongoing research in this field has already

established the involvement of deregulation of cyclin D1 in the pathogenesis of many different types of cancer including for instance breast cancer and ovary cancer, consistent with the fact that increased cell proliferation is a hallmark of virtually al cancers. Additionally, it has also been found that polymorphisms in the cyclin D1 gene was tied to increased risk of developing cancer, such as colorectal cancer. It is also known, however, that for each particular incidence of cancer the involvement of cyclin D may depend on which other oncogenes are involved.

As illustrated in the case of cyclin D, cancer is indeed a complex group of diseases in the sense that no single oncogene is known to be responsible for all incidences of a single type of cancer. However, a particular defect causing effects such as increased cell division/proliferation, cell migration and angiogenesis, which are central processes in the pathogenesis of cancer, must be assumed to play a role in at least a fraction of all incidences of most types of cancer. There is therefore also some expectation of success when applying the analogues of the invention in cancer treatment in general.

Further support of the use of analogues according to the invention in cancer treatment may be found in studies on their effects in human melanoma cells. These studies have been conducted by a group of researchers in Chile in collaboration with the inventors. Results from the studies are presented in the enclosed manuscript by M. Kurte et al. with the title: A synthetic Peptide Homologous to the Functional Domain of Human Interleukin 10 Downregulates the Expression of the MHC class I Alpha Chain and TAP 1/2 in human Cells. This manuscript has been accepted for publication in Journal of Immunology, but unfortunately the inventors have only been able to provide part of the manuscript. In brief, these studies have shown that IL-10/IT9302 also has an indirect cancer-restrictive effect by stimulating Natural Killer Cell-mediated tumor lysis. From

figure 5, in particular, it appears that IT9302 induces a higher sensitivity to lymphokine activated killer cell (LAK) mediated cytotoxicity in a melanoma cell line. This effect was seen even when low doses of the analogue, such as 1 ng/ml, were used.

With respect to our previous argumentation for the enablement of claims directed to methods of treatment, which was backed by new information from the inventor in declaration form there is no indication at all in the Office Action that this material has been fully considered by the Examiner. We have enclosed an amended version of the already submitted declaration (Declaration A), which provides a review of the current knowledge on IL-10's regulation of proinflammatory cytokines and their involvement in cancer and in psoriasis. The declaration also describes the role of the transcription factor NF-κB in inducing proinflammatory gene expression. Experimental results presented at a meeting in Alexandria in Egypt demonstrating the regulation of inflammatory cytokines by IL-10 and its analogues via NF-κB are included. Furthermore, treatment of cells with the analogues resulted in induction of the tumor suppressor protein p53 resulting in apoptosis.

A second declaration (Declaration B) reviews a study documenting the ability of IL-10 analogues to regulate cell proliferation in a carcinoma cell line. The results of this study were presented at a meeting in Maui, Hawaii. Declaration C contains the material previously filed as a copy of a poster from a conference in Turin, Italy supporting the use of synthetic analogues in cancer treatment.

Finally, the Examiner's attention is respectfully directed to Ex parte Rubin, 5 USPQ2d 1461 (BPAI 1987). The claim in that case read as follows

> 1. A method for improving the effectiveness of interferon in the treatment of neoplastic conditions accompanied by an increase of the patient's serum tyrosinase level

> substantially above normal, comprising administering an agent for inhibiting tyrosinase in an amount sufficient to substantially inactivate the tyrosinase in the serum of the patient being treated with interferon, and administering the interferon during the time in which the tyrosinase is being inhibited by said agent.

It thus encompassed treatment of any neoplastic condition characterized by elevated tyrosinase levels. Despite the fact that it was not limited to a specific cancer, the Board held that it was fully enabled.

The Rubin decision appears relevant not only to the instant rejection of claim 49, but also to the rejection of claim 87, which refers to IL-10 levels.

Conclusion

It is believed that the case is now in condition for allowance. However, if the Examiner maintains the rejection stated in OA §2a, then the next rejection cannot be made final, as OA §2a failed to consider the timely filed August 7 supplemental response.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

By: _____
Iver P. Cooper
Reg. No. 28,005

Enclosures
-Declarations A-C
-Article by Pahl
-Manuscript by Kurte et al.

624 Ninth Street, N.W.
Washington, D.C. 20001
Telephone: (202) 628-5197
Facsimile: (202) 737-3528
IPC:lms G:\ipc\n-q\Plou\GronhojLarsen2\pto amend4.wpd

DECLARATION 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Christian Grønhøj Larsen and Borbala Gesser
S rial no.	:	09/101,825
Filed	:	July 17, 1998
For	:	Synthetic IL-10 Analogues
Examiner	:	F. Hamud
Art unit	:	1646

Declaration of Borbala Gesser

1. I, Borbala Gesser, Pilegårdsvej 233, DK-8361 Hasselager, Kolt, Denmark, in my capacity as an assistant professor at Marselisborg Hospital, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have compiled the following information regarding the role of inflammatory cytokines in psoriasis and cancer. The information is derived from articles published in peer reviewed scientific journals as indicated by the list of references below as well as from work c nducted in our laboratory. In doing so, my intention is to summarise what will be known to a person skilled in the art with respect to inflammatory cytokines in cancer and psoriasis.

4. Inflammatory cytokine´s effect on cancer: Inflammatory diseases are characterized by cells producing high concentration of inflammatory cytokines, specially IL-8, MCAF and TNF-α.
Cancer cells like melanomas produce IL-8, MCAF and serum levels of IL-8 correlate with the amount of tumour (Scheiber et al., 1995). Melanoma cells stimulated with IL-1α or TNF-α produced 10 fold higher quantities of IL-8 than normal human melanocytes (Zachariae et al., 1991).
IL-8 induces migration of melanoma cells (Ji Ming Wang et al., 1990) and IL-8 is involved in melanoma metastasis (Rakesh K. Sing et al., 1994).
Also human breast carcinoma cells respond chemotactically to IL-8 and MCP-1 and this chemokines might play a role in breast carcinoma cell migration (Sara J. Young et al., 1997).
Macrophages play a key role in inflammatory and tumour angiogenesis by releasing growth factors and monokines (IL-1, IL-6, IL-8, TNF-α, prostaglandins (Cord Sunderkøtter et al., 1994).

5. IL-10´s regulation of cancer: IL-10 release from transfected melanoma cells inhibited IL-1β, TNF-α, IL-6, proteinase matrix metalloproteinase-9 and vascular endothelial growth factor production from tumour associated macrophages. IL-10 thereby inhibited angiogenesis (Suyun Huang et al., 1996).
In a murine model of breast cancer IL-10 transfected tum ur cells implanted subcutaneosly in immunoc mpetent BALB mice showed that tumour growth measured by

mean diameter was significantly inhibited, after 17 days, compared with non transfected cells (Namita Kundu et al., 1996).

6. IL-8´s effect on psoriasis: Interleukin-8 promotes epidermal cell proliferation Andrea Tuschil et al., 1992. It is also documented that psoriatic epidermis express high levels of IL-8 (Giustizieri M. L., et al., 2001).
Response of psoriasis to IL-10 is associated with suppression of IL-8/CXCR2 (IL-8 receptor) pathway and normalisation of keratinocyte maturation (Reich K et al., 2001).
Tumour suppresser protein p53 is found in decreased amount in involved psoriatic skin compared with uninvolved skin and normal skin. Antipsoriatic drug Tacrolimus was able to suppress IL-8 and induce p53 transcription (Michel et al., 1996).

The importance of suppressing inflammation in psoriasis or cancer was further proved by Hudson et al., 1999, who showed that "A pro-inflammatory cytokine (MIF) inhibits p53 tumour suppressor activity". In a variety of tumours, p53 is functionally inactivated, but the gene remains intact (Moll, U.M., 1992, 1995). Thus, identification and characterisation of novel regulators of p53 activity may have direct consequences for understanding the etiology of multiple tumour types.

7. NF-κB activation.
The transcription factor NF-κB controls the induction of several inflammatory genes like the IL-1β induction of IL-8 and NF-κB also controls apoptosis (Baerle et al.,1996, Barkett et al., 1999, Kajino S et al., 2000). The binding site of the p53 promoter contains response elements for NF-κB and the expression of p53 can be induced by NF-κB/p65 and TNF-α (Wu H & Lozano 1994, Pahl HL 1999).
NF-κB is an inactive protein complex in the cytoplasm containing hetero or homodimeric complexes of p65/p65, p65/p50 or p50/p50 proteins. These proteins are bound to IκB-α in inactive form. Following inflammatory activation IκB-α gets phosphorylated and degradated and the NF-κB proteins translocate to the nucleus and thereby activate DNA promoter sites (Gilmore TD 1999, Henkel et al., 1993).
NF-κB DNA binding is highly up-regulated in tumor cells compared with normal cells (Denvalaraja et al., 1999, Girii DK. and Aggarwall BB. 1998)

Interleukin-10 signalling blocks NF-κB activity at two levels: 1. through suppression of IκB-α phosphorylation and 2. through inhibition of NF-κB p65/p50 DNA binding (Schottelius et al., 1999). Interleukin-10 also stabilizes Iκ-Bα in human monocytes (Shams et al., 1998).

NF-κB activation in inflammation is biphasic: DNA binding activity occurs once on the onset of inflammation, early, and later after 48 hours at the resolution of inflammation. Time study analysis of cytokine and apoptosis related gene expression in carrageenin-induced pleurisy showed that for example TNF-α was induced at 6 hours while bax (apoptosis regulator protein) reached maximum at 48 hours. Inhibition of NF-κB during the resolution of inflammation protracted the inflammatory response and prevented apoptosis (Toby Lawrence et al., 2001). In conclusion, proper NF-κB regulation is essential for induction of apoptosis.

8. To be able to address which type of cancer can be suppressed by IL-10 or IT9302:

We refer to two published abstracts:

"The C-terminal part of IL-10 is regulating proliferation and apoptosis in hepato-cellular carcinoma cells through a p53 mechanism" B. Gesser & C. G. Larsen.
This was presented as a poster at an international scientific meeting, "The Cytokine Odyssey, 2001", 8th-11th November, 2001 Maui, Hawaii. The abstract is published in Journal of Leukocyte Biology, Supplement 2001, 166, page 56. This findings are enclosed in a separate declaration.

"The role of an IL-10 analogue IT9302 in regulating proliferation and apoptosis in Hepato-cellular carcinoma cells."
B. Gesser, C. Vestergaard, M. Rasmussen, C. Johansen, N. Kirsteijn, L. Iversen, K. Kragballe, C. Grønhøj Larsen.
This was an oral presentation at the World Congress in Alexandria, Egypt, 13th-15th September, 2003. The abstract is published in " World Congress of The African Federation of The International College of Surgeons, National Liver Institute, Menoufiya University, The Egyptian Society of Hepatology, Gastroentology and Infectious Diseases in Alexandria" September 13th-15th, 2003, Program & Abstracs.

Abstract presented in Alexandria:

"IL-10 has inhibitory effect on growth of cancer cells. Human IL-10 has been shown (Berman et al., 1996) to induce long lived immune response added at high doses to mice bearing established sarcomas, melanomas or colorectal carcinomas. We examined if IT9302, an IL-10 agonist and homologous to the C-terminal domain, also is responsible for the anti-proliferative effect of IL-10.
We wished to examine the possible relation between IL-10 and the tumour suppressor protein p53 in regulating cell proliferation. Hepato-carcinoma cell lines HepG2 (p53 wild type), Hep3B (p53 deleted) and PLC/PRF5 (p53 mutated) were stimulated daily for 7 days with 5 µg/ml of IT9302. We saw a significant anti-proliferative effect, selectively in Hep G2 cells (day 5).
As IL-10 is known as a regulator of IL-8 production in monocytes, we additionally studied how the production of IL-8 was regulated in Hepato-carcinoma cells, when exposed to high doses of IL-10 or IT9302, relevant to cancer situations.
Results: 5µg/ml of IT9302 induced IL-8 at day 2 in HepG2 cell. This was accompanied by the induction of the tumour suppressor protein p53, at day 3. The IL-8 production was then blocked from day 3 and the following days. In PLC/PRF5 cells (p53 mutated) IL-8 production was elevated slightly by IT9302 but there was no induction of p53. There was no change in IL-8 production in Hep3B cells. In HepG2 cells p53 activity caused apoptosis from day 5, shown by detection of PARP cleavage from day 4 and 5.
The IL-8 promoter site is a NF-kappaB activated gene. We confirmed by DNA binding studies that IT9302 and IL-10 blocked NF-kappaB binding to the IL-8 "kappaB" site from day 3. At the same time, binding to the p53 "kappaB" motif was induced. This proved to be a highly important event for the induction of p53 protein. In control experiments we used an IkappaB phosphorylation blocking agent, sodium salicylate (20 mM/ml) in combination with IT9302 (5µg/ml). This resulted in further induction of p53 wild type protein from day 3. Sodium salicylate alone did not induce p53 protein.
In conclusion: Pro-inflammatory cytokines inhibited p53 tumour suppressor activity in a mouse embryonic cell line shown by Hudson et al., 1999. According to us, in Hepatocarcinoma cells the anti-prolifarative effect of IT9302 (5 microgram/day) was

dependent on wild type p53 expression (HepG2 cells) and blocking of IL-8 production. The main target for the IL-10 analogue IT9302 is to suppress the level of inflammatory cytokines, especially IL-8 and thereby induce the tumour suppressor protein p53."

Transcriptional Cross Talk between NF-kappaB and p53.

P53 has a key role in the negative regulation of cell proliferation and in the suppression of transformation and tumorigenesis. A proinflammatory cytokine MIF (macrophage migration inhibitory factor) was identified which was capable of functionally inactivating p53 (Hudson et al., 1999). This pointed to a possible link between inflammation and tumorigenesis.
P53 and NF-κB/p65 are activated by various genotoxic agents and they suppress each other's ability to activate transcription (Ikeda et al., 2002). Theses results directly indicated that there is a direct interaction between these two transcription factors. Further it was shown that only the wild type p53 was able to suppress NF-kappaB/p65 activity. Finally cells derived from Rel A (p65) knockout mice showed enhanced p53 activity, in cellular response to genotoxic stress.
Other studies showed that p53 was able to inhibit Rel A (p65) transcription activity (Webster GA et a., 1999) while p53 activity was repressed by NF-κB/p65 but not by NF-κB/p50. Also endogenous p53 transcriptional activity was repressed by TNF-α activated NF-κB/p65. Following salicylate treatment nuclear translocation of NF-κB/p65 was inhibited and enhanced TNF-α induced apoptosis occurred in human pancreatic cancer cells (McDade T, et al., 1999).
This is in agreement with our results from HepG2 cells. In control experiments we user the Iκ-B phosphorylation blocking agent, sodium salicylate in combination with IT9302. This supported the induction of p53 protein in HepG2 cells. Sodium salicylate alone did not induce p53 protein."

NF-κB activation is associated with the onset of pro-inflammatory gene expression. During the resolution of inflammation anti-inflammatory and apoptotic genes are activated by NF-κB (Lawrence et al., 2001). The presence of NF-κB p50/p50 homodimer during the resolution of inflammationis not surprising considering the role of p50/p50 in the repression of pro-inflammatory gene transcription. In our results p50 was identified as the predominant NF-κB protein after 3 days stimulation either with IT9302 or IL-10. NF-κB is known to regulate p53 expression, however in addition p50/p50 homodimer positively regulate the p53 promoter (Wu & Lozano 1994, Sun X. et al., 1995, Kirch HC. et al., 1999). It is possible that p53 and NF-κB p50/p50 cooperate to regulate Bax expression and apoptosis.

9. IL-10 and IT9302 mediated apoptosis through binding to the IL-10R2 and subsequent specific regulation of NF-κB in HepG2 cells.

IL-10 R2 is expressed in all cells except the brain, Spencer et al., 1998. Lately IL-10 R2 was shown as a shared receptor between IL-10 R1 and IL-21 R1. HepG2 cells express IL-21 R1 and IL-10 R2, Dumoutier et al., 1997. IL-10 R2 was also shown as a co receptor to IL-22 R1 Kotenko et al., 2001. The abundance of IL-10 R2 in all tissues may point to its central importance for regulating cellular functions.

12. We next set out to investigate if any of the synthetic analogues were able to regulate NF-κB binding to DNA. We selected two IT9302 Synthetic analogues from the group of 35, which were tested before for their ability to induce IRAP in purified monocytes or U937 monocytic cells.

Analogues tested: A_2 H-1BUA-YMTMKIRN-OH (1BUA=1-butylamin)
A_3 H-MEA-YMTMKIRN-OH (MEA=methoxyethylamin)

Results from these experiments are collected in a poster, which is enclosed in a separate delaration:

> Novel Synthetic analogues of IL-10 regulate the binding of NF-kappaB complexes to p53 and IL-8 kapaB motifs. Christian Grønhøj Larsen, Claus Johansen, Lars Iversen, Arne Holm, Borbala Gesser Cytokines and Interferons 2002, Turin, October 6-10, 2002.

Conclusion: IL-10 as well as the IL-10 analogues significantly modulates the binding of NF-κB to DNA supporting the practicability of using the IL-10 analogues in the treatment of cancer. Furthermore, novel IL-10 analogues, which are modifications of IT9302 may have improved IL-10 like effects and may also exhibit enhanced stability compared to IT9302.

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12. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 14. dec. 2003 Signature: Borbala Gesser

Borbala Gesser

DECLARATION 12.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Christian Grønhøj Larsen and Borbala Gesser
Serial no.	:	09/101,825
Filed	:	July 17, 1998
For	:	Synthetic IL-10 Analogues
Examiner	:	F. Hamud
Art unit	:	1646

Declaration of Borbala Gesser

1. I, Borbala Gesser, Pilegårdsvej 233, DK-8361 Hasselager, Kolt, Denmark, in my capacity as an assistant professor at Marselisborg Hospital, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have been conducting the following studies to demonstrate the effect of the IL-10 analogue IT9302 in cultured hepato-cellular carcinoma cell. The following results are able to address the mechanisms involved in growth regulation by IL-10 and IL-10 analogues. The information is derived from poster originally presented at an international scientific meeting, "The Cytokine Odyssey, 2001" 8th-11th November, 2001 Maui, Hawaii. The abstract is published by the title "The C-terminal part of IL-10 is regulating proliferation and apoptosis in hepato-cellular carcinoma cells through a p53 dependent mechanism." B. Gesser & C. G. Larsen in the Journal of Leukocyte Biology, Supplement 2001, 166, page 56.

4. Introduction: Interleukin 10 (IL-10) has pro-apoptotic activity on malignant cells. The effect of IL-10 is partly confined to the C-terminal domain. We examined if IT9302, an IL-10 agonist and homologous to the C-terminal domain, is also responsible for the anti-apoptotic activity of IL-10. We have earlier observed that IT9302 induced the proportion of normal human monocytes expressing apoptosis; IT9302 opposed the apoptosis-inhibiting effect of IFN-γ. We wished to study if there is a possible relation between IL-10 and the tumour suppressor protein p53 with respect to the regulation of cell proliferation. As IL-10 is known as a regulator of IL-8 production in monocytes, we additionally studied how the production of IL-8 is regulated in Hepato-carcinoma cells when exposed to high dosis of IL-10/IT9302, relevant to cancer situation.
In a study of the effect of 2.5 µg/ml of IT9302 on three hepato-carcinoma cell lines, HepG2 (p53 wild type), PLC/PRF5 (p53 mutated) and Hep3B (p53 deleted), we saw a significant anti-proliferative effect, selectively on HepG2 cells.

5. Methods: Cell lines were purchased from the European Collection of Cell Cultures, Salisbury, and Wiltshire, SP4 OJG, UK. Human hepatocarcinoma cells: HepG2 (p53 wild type), PLC/PRF/5 (p53 mutated) and Hep3B (p53 deleted) were cultured in DMEM (Gibco Cat. no 61965 containing 2 mM glutamine) addad 25 mM Hepes and 10 % FCS (Gibco. 160000 44 USA origin) and Penicillin 50 IU per ml, Streptomycin 50 IU per ml and

Gentamycin 2.5 µg/ml. Cells were cultured to exponential growth and trypsinated with 0.1% trypsin added 0.2 % EDTA. Cells were thereafter divided in portions of 1.5×10^6 cells/ T25 bottle added 4 ml culture medium and were either stimulated once with 0 and 2.5 µg/ml of IT9302 or daily with IT9302 in concentrations of 0, 0.05, 0.5 and 5 µg/ml in seven parallel experiments. Cells were either cultured for 5 days or for 7 days. The Synthetic peptides were resolved and diluted in sterile isotonic salt 30 minutes before the stimulation occurred. Cells were collected and the number of cells was counted for every day and cells and supernatants were kept at -80 ^{0} C until further analysis.
Determination of p53 production in three parallel experiments of hepato-carcinoma cells were according to the protocol from Pantropic p53 Rapid format ELISA Kit (cat. no. QIA26, Calbiochem, Amersham, DK). IL-8 secretion was measured by a modified version of ELISA, previously described (Ko Y et al., 1992) and monoclonal anti-human IL-8 antibody (WS-4, Dainippon Pharmaceutical, Osaka, Japan).
For Western Blotting studies cells were collected in gel lysis buffer (Cells JE et al., 1989) and 50 µg of protein was applied per well on 10 % SDS-PAGE gels.
Western blotting of IκB-α was with polyclonal rabbit IκB-α antibody (C21-): sc-371, Santa Cruz, Biotechnology, US) and and swine anti-rabbit-HRP (cat.No P217, Dako, Denmark). Western blotting of PARP was with polyclonal rabbit PARP antibody (Boeringer Mannheim, Cat. no 1835 238, Denmark) and swine anti-rabbit-HRP. The binding was developed by enhanced chemiluminescence's activation (cat. n. RPN 2106, ECL, Amersham, DK) and immunostaining was detected by exposing a film (Kodak X-Omat-S) for 30 sec.

6. Results: An oligopeptide IT9302, with homology to the C-terminal part of the mature IL-10, is able to regulate cell proliferation in a hepato-carcinoma cell line, HepG2 (p53 wild type), while cell proliferation in PLC/PRF5 (p53 mutated) and Hep3B (p53 deleted) were unaffected by IT9302 (Fig. 1).

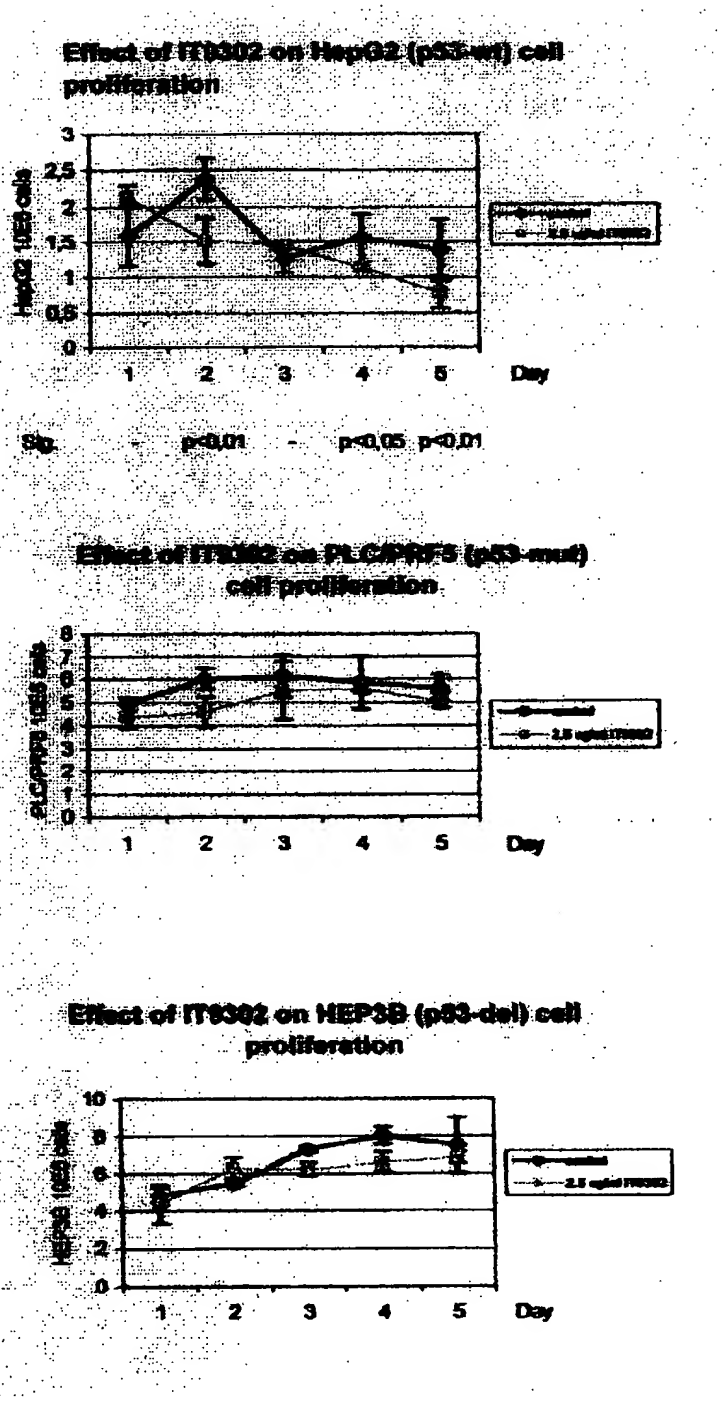

Effect of IT9302 on HepG2 (p53-wt) cell proliferation
HepG2 (IEB cells
3
2,5
2
1,5
1
0,5
0
1 2 3 4 5 Day
Sig. - p<0,01 - p<0,05 p<0,01
Effect of IT9302 on PLC/PRF5 (p53-mut) cell proliferation
8
7
6
5
4
3
2
1
0
1 2 3 4 5 Day
Effect of IT9302 on HEP3B (p53-del) cell proliferation
10
8
6
4
2
0
1 2 3 4 5 Day

Fig. 1

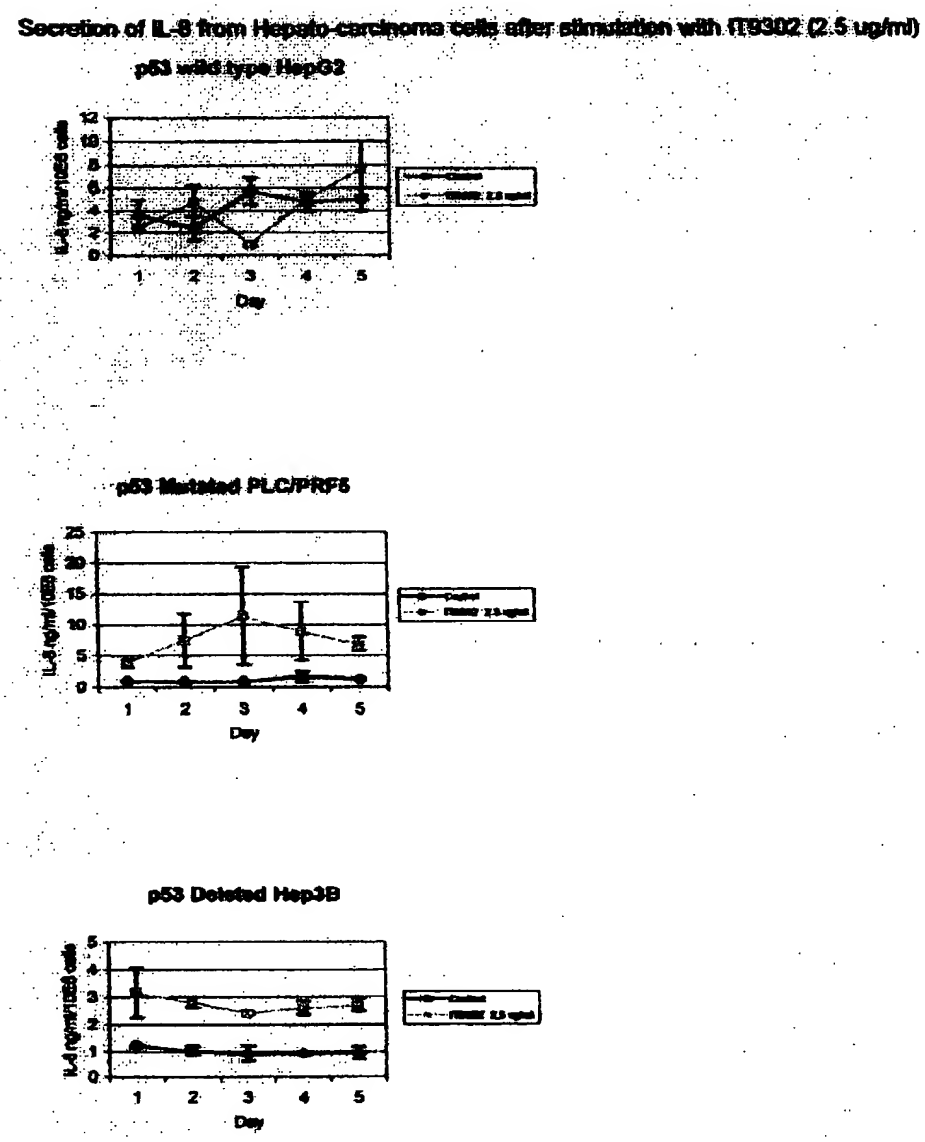

Secretion of IL-6 from Hepato-carcinoma cells after stimulation with IT9302 (2.5 ug/ml)
p53 wild type HepG2
Day
p53 Mutated PLC/PRF5
Day
p53 Deleted Hep3B
Day

Fig. 2

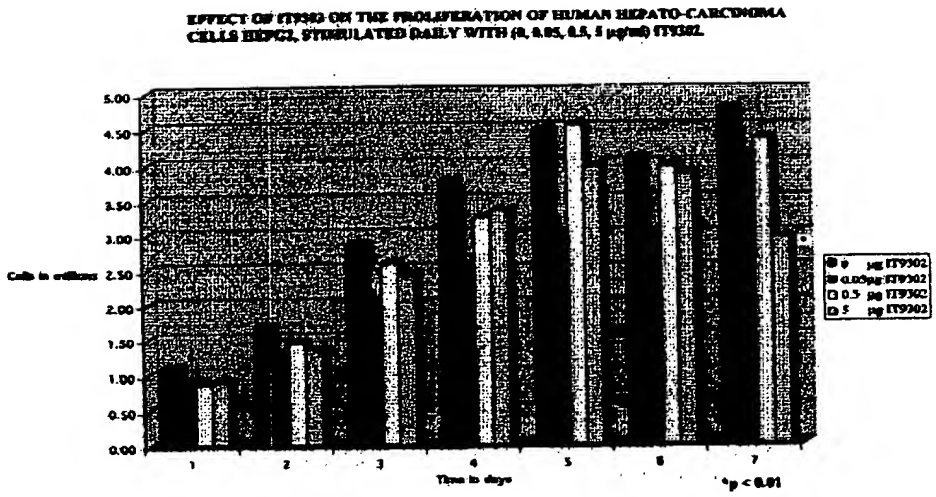


Fig. 3

HepG2 cells pulse stimulated with 5 µg/ml of IT9302 showed significant decreased proliferation at day 7 (Fig. 3).
HepG2 cells treated only once with 2.5 µg/ml of IT9302 induced their IL-8 secretion at day 2 but clearly inhibited IL-8 production at day 3 (Fig. 2). This was accompanied by the relative induction of the intracellular p53 production day 3 (Fig. 4A).There was no induction of p53 in PLC/PRF5 cells (Fig. 4B) and the induction of IL-8 was present only in PLC/PRF5 cells (Fig. 2) but not in the Hep3B cells.
Daily stimulation with 5 µg/ml of IT9302 in HepG2 cells induced IL-8 at day 2, while this was suppressed from day 3. Daily stimulation with 5 µg/ml of IT9302 induced p53 at day 3 and 4 (Fig. 5A and Fig. 5B). The relative induction of p53 was higher than the relative induction of IL-8 from day 3 and the following days (Fig. 6).

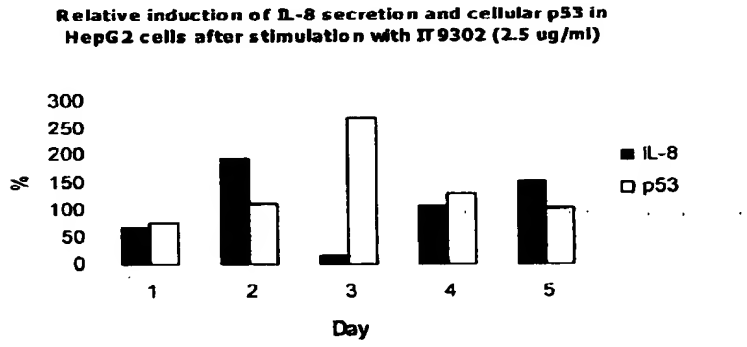


Fig. 4A

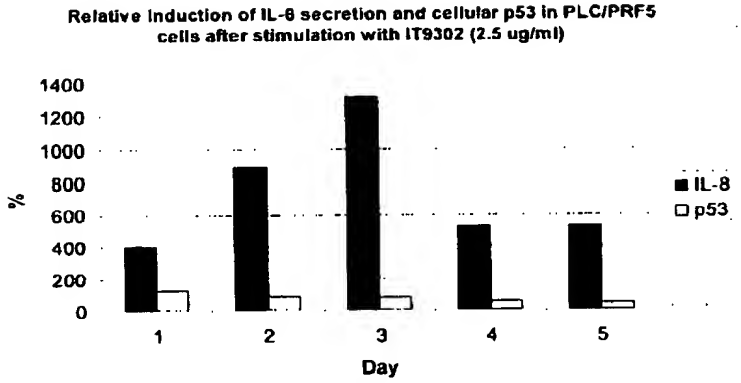


Fig. 4B

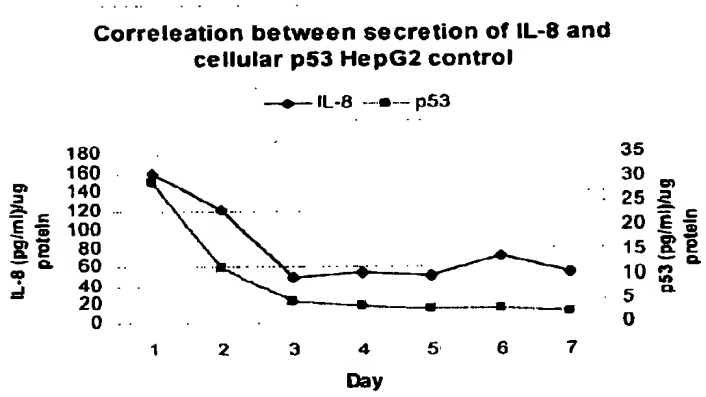


Fig. 5A

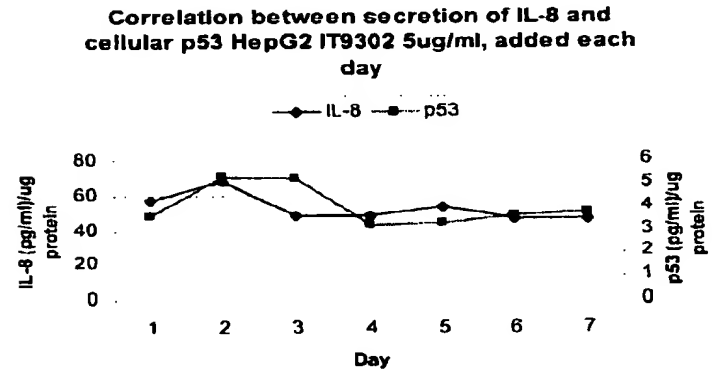


Fig. 5B

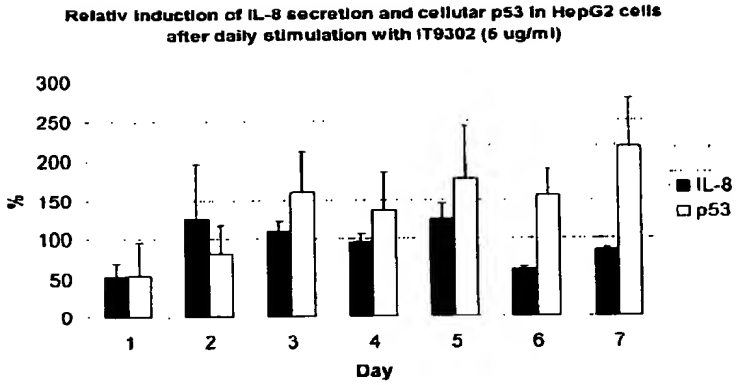


Fig. 6

In the daily stimulated HepG2 cells, Iκ-Bα was down regulated at day 1, but new synthesis was observed from day 2 and 3 compared with the non stimulated cells (Fig.7).
The active form of PARP 116 kDa protein (Poly-(ADP-ribose)-polymerase) was detected and induced at days 4 and 5 in the non-stimulated cells. The stimulated HepG2 cells expressed the PARP cleaved form at days 4 and 5, which results in inactivation of its function for DNA replication and indicates apoptosis (Fig. 8).

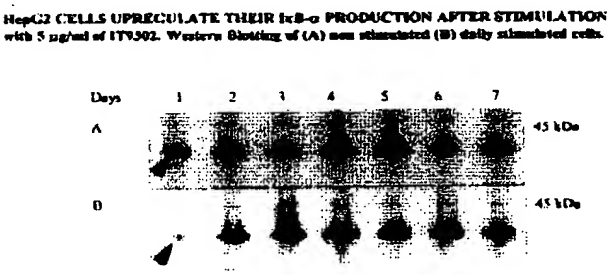


Fig. 7

DETECTION OF PARP CLEAVAGE BY AN ICE-LIKE PROTEASE, IN HEPG2 CELLS shown by Western Blotting. A. Non stimulated B. Daily stimulated with 5 µg/ml of IT9302.

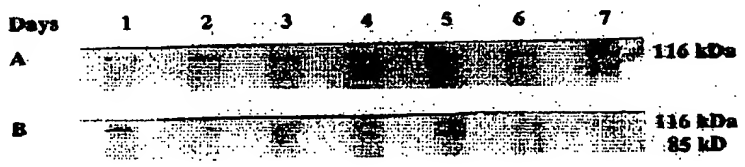


Fig. 8

7. Conclusion: The C-terminal part of IL-10 regulates cell proliferation in HepG2 cells, a function, which may be dependent on p53 (wild type) and the induction of NF-κB (followed by IL-8 induction).
The binding site of the p53 promoter "κB motif" contains response elements for NF-κB (Donehower et al., 1993). The p53 promoter binds the hetero-dimer complex of NF-κB (p65/p50) (Hellin et al., 1998). IL-8 belongs to the group of genes which preferentially bind NF-κB in the form of Rel homo-dimers (p65/p65) (Mukaida et al., 1990, Yamamoto et al., 1992).
Th affinity of the p65/p65 and p65/p50 protein complexes to IL-8 respectively p53 "κB" motifs, are different. This could explain why IL-8 is induced before p53 (Yamamoto et al., 1992). IκB-α is also a NF-κB regulated gene (Pahl HL 1999), which was confirmed by our results.
Newly synthetized IκB-α, augmented by IL-10/IT9302, enters the nucleus and will uncouple active NF-κB from its DNA binding site (Zabel et al., 1990, 1993). IκB-α inhibits only the DNA binding of p65 (Ghost et al., 1990, Gilmore et al., 1996). The uncoupling of NF-κB from DNA is essential, since non-stimulated HepG2 cells did not exhibit apoptosis.

HepG2 cells express the IL-10 R2 (CRF2.4) but not the IL-10 R1. We therefore speculate that the apoptotic effect of IL-10 and IT9302 is mediated through IL-10 R2.

8. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful fase statements may jeopardise the validity of application or any patent issued thereon.

Dated: 17. dec. 2003 Signature: Borbala Gesser

Borbala Gesser

DECLARATION C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Christian Grønhøj Larsen and Borbala Gesser
Serial no.	:	09/101,825
Fil d	:	July 17, 1998
For	:	Synthetic IL-10 Analogues
Examiner	:	F. Hamud
Art unit	:	1646

Declaration of Borbala Gesser and Christian Grønhøj Larsen

1. I, Borbala Gesser, Pilegårdsvej 233, DK-8361 Hasselager, Kolt, Denmark, in my capacity as an Assistant Professor at Marselisborg Hospital, DK-8000 Aarhus C, Denmark, and I, Christian Grønhøj Larsen, Rislundvej 7, DK-8240 Risskov, Denmark in my capacity as an Associate Research Professor at Marselisborg Hospital, Dk-8000 Aarhus C do state and declare as follows:

2. We are among the named inventors of the above-captioned patent application. We believe that we are persons skilled in the art to which the above-captioned application pertains.

3. We have been conducting the following studies demonstrating the effects of IL-10 and IL-10 analogues in cultured cell lines. The studies were originally presented at an international scientific meeting, "Cytokines and Interferons 2002", October 6-10, 2002, Lingotto Congress Center, Turin (Italy), in the form of a poster titled "Novel Synthetic analogues of IL-10 regulate the binding of NF-kappaB complexes to p53 and IL-8 kappaB motifs." The poster is published in Journal of Interferon & Cytokine Research 2002, vol.22, Supplement1, page S-114.

4. Introduction: NF-κB is a transcription factor involved in the regulation of inflammation and growth in both benign and malignant cells (Gilmore et al., 1996). We have recently shown that in hepato-carcinoma cells both IL-10 and IT9302 (**-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn**) , a nona-peptide homologous to IL-10´s COOH terminal domain, is regulating NF-κB complexes by inducing NF-κB 50 and IκB-α and thereby inducing apoptosis (Gesser et al., 2002). In this study we tested whether IL-10, IT9302 and two novel IL-10 analogues are able to regulate NF-κB binding to specific "κB motifs" in m nocytes as well as in a monocytic cell line U937. We constructed novel IL-10 analogues by modifying the amino-acid composition of IT9302, substituting single amino acids with natural or non-natural amino-acids.
IL-10 is known for inhibiting Nuclear Factor κB activation (Wang et al., 1995) and for stabilizing Inhibitory κB-α in monocytes (Shames et al., (1998). IL-10 is also blocking κB kinase activation and induced NF-κB p50/p50 in the monocytic cell line U937 (Schottelius et al., 1999).
Specific "κB motifs" are identified for IL-8, Mukaida et al., (1990), Harant et al., (1998) as well as for p53, Kirch et al., (1999).
We find that both IL-10 and IT9302 (in equimolar concentrations) suppress NF-κB binding to DNA, around 10 to 30 minutes after stimulation (Fig. 1) .

We further find that IL-10, IT9302 and novel analogues also modify the binding of NF-κB to the IL-8 "κB motif" as well as to the p53 "κB motif" oligonucleotides.

Target genes for NF-κB and their "κB" motifs.

human IL-8 promoter	binding proteins	Ref.
3' GGAATTTCCTC 3'	Rel A homodimer (p65/p65)	Mukaida et al., (1990) Harant et al., (1998)
human p53 promoter		
5' TGGGGTTTTCCCC 3'	NF-κB (p50/p65) heterodimer	Kirch et al. (1999)
mouse p53 promoter		
5' TGGGACTTTCCCC 3'	(p50/p65) heterodimer	Wu H. and G. Lozano (1994)

Inhibitors of NF-κB/Rel proteins are

Class I	Nfkb1 Nfkb2	p105 p100	Gilmore et al., 1996.

and IκB proteins which contain ankyrin repeats.

IκB-α 37 kD	Inhibits only DNA binding of p65	Ghosh et al., 1990. Gilmore et al., 1996.

5. Methods: Human monocytes or U937 monocytic cells were cultured in $6x10^6$ cells / 3 ml medium, DMEM added 25 mM Hepes and 5% FCS, (Hyclone, logan, UT). Cells were then either non-stimulated or stimulated for 24 hours with 10 ng/ml of rIL-10 or equimolar amounts of IT9302 or its analogues. The following day cells were stimulated with rIL-10 or analogues once more for 30 minutes before stimulation with 30 ng/ml of IL-1β for 1 hour. Peptides were designed and synthesized by Professor Arne Holm, Royal Veterinarian Academy, Copenhagen. The peptides were dissolved in sterile saline before used.
Cells were washed in ice cold PBS and nuclear proteins were isolated as previously described (Johansen et al., 2000). Electrophoretic mobility shift assay (EMSA) for NF-κB binding was performed with 2 μg of nuclear protein added 2 μl of 32P-labelled NF-κB probe:
IL-8 consensus NF-κB 5´-CAAATCGGGAATTTCCTC-3´ or Promega consensus NF-κB 5´AGTTGAGGGGACTTTCCCAGGC-3´ were purchased from AH diagnostic, Aarhus, Denmark.
Supershift reactions included anti-NF-κB/p65 antibody and anti-NF-κB/p50 antibody (Santa Cruz, CA).

6. Results: We compared two synthetic IL-10 analogues A2 and A3, which are nona-peptides containing non-natural amino acids, with IL-10 and IT9302 for their ability to block NF-κB binding to specific "κB motifs" (Fig.2-5).
In a system of human monocytes, IT9302 and A3 more efficiently suppressed NF-κB binding to the IL-8 "κB motif" than to the p53 "κB motif" (Fig.2 and 3). Also, in repeated experiments, A3 was more potent than both recombinant IL-10 and the nonapeptide IT9302. In fact A3 suppressed the binding to levels below that of the negative control.
In U937 cells, the relative NF-κB binding was also suppressed by IL-10, IT9302 as well as the two new analogues (equivalent to 10 ng/ml of rIL-10) in a d se dependent manner.

A3 and A2 analogues were more efficient than IL-10 and IT9302 with respect to inhibiting the binding to the IL-8 "κB motif" while A3 was most efficient with respect to inhibition of the binding to the p53 "κB motif" in U937 cells.

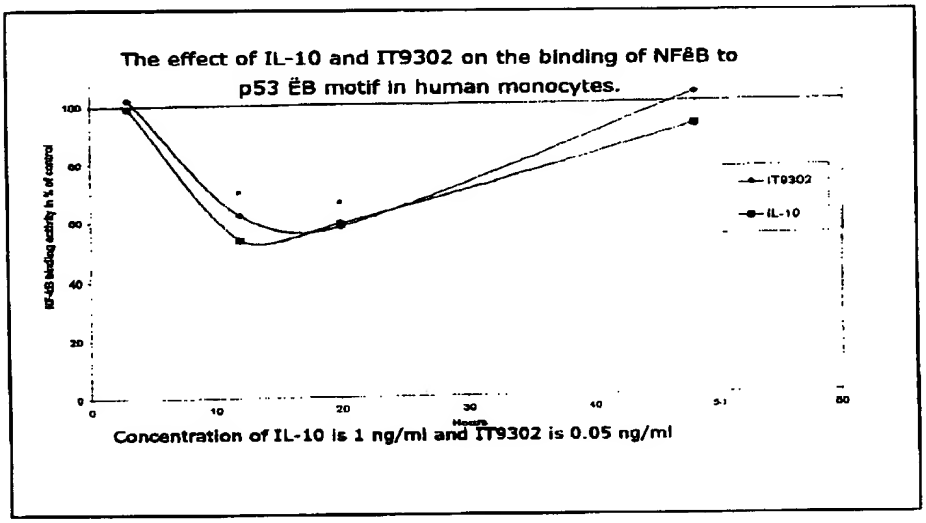


FIG. 1

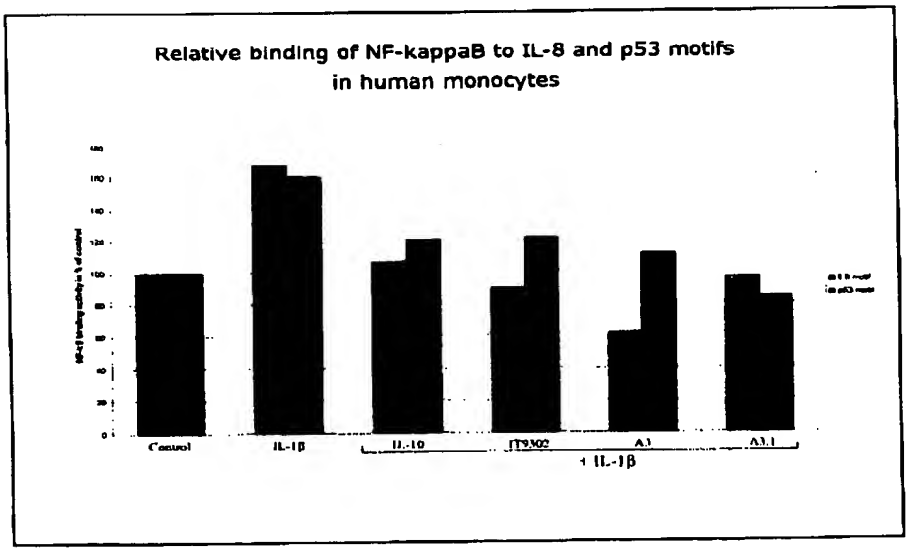


FIG. 2

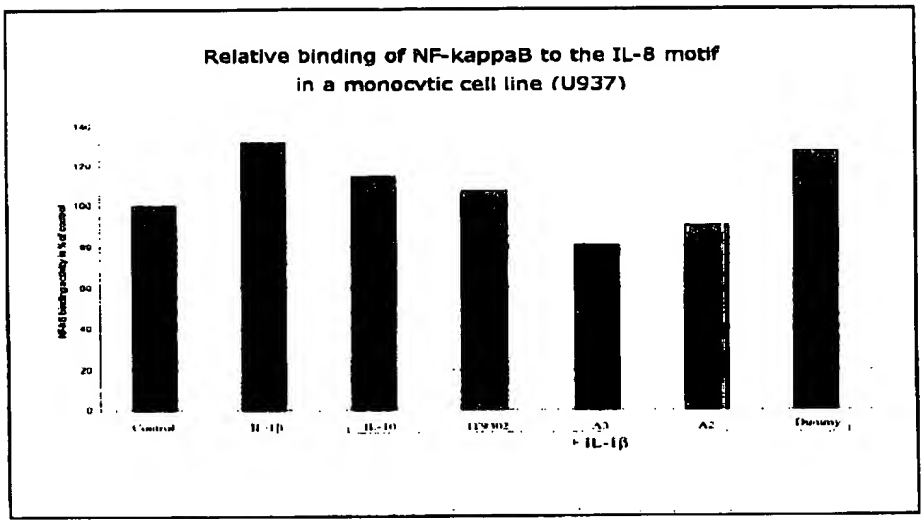

Relative binding of NF-kappaB to the IL-8 motif
in a monocytic cell line (U937)
+ IL-1β

FIG. 3

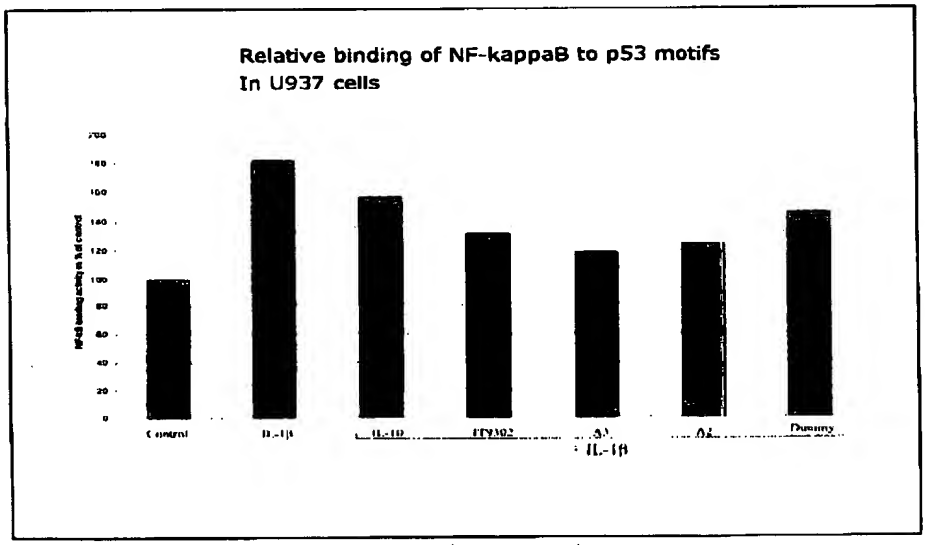

Relative binding of NF-kappaB to p53 motifs
In U937 cells
+ IL-1β

FIG. 4

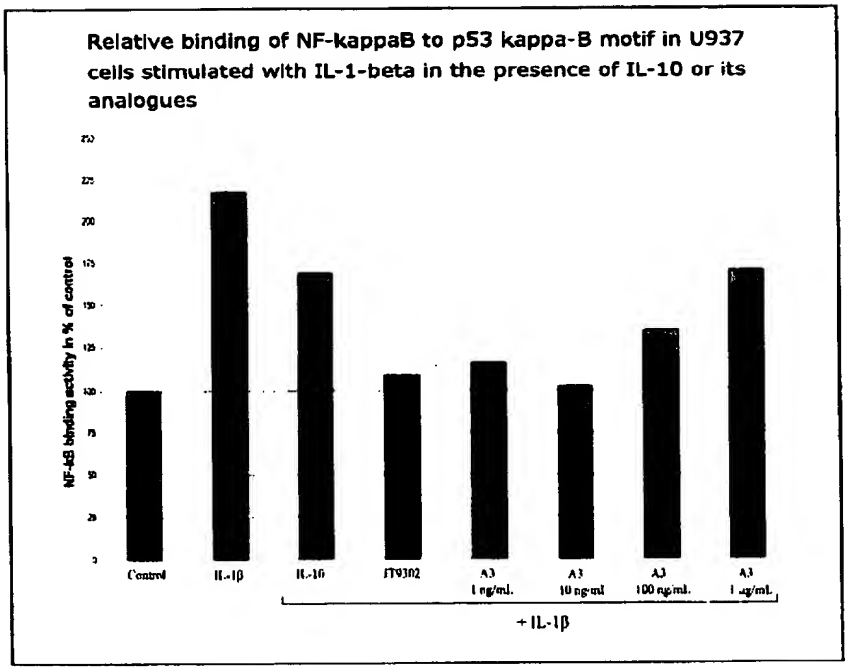


Fig. 5

7. Discussion and conclusion: IκB-α can specifically blocksNF-κB/p65 binding to DNA Ghosh S., Baltimore D. (1990) and thereby block binding of p65/65 homodimers to IL-8 "κB motif". In our hands, effective de novo synthesis of IκB-α appears to be dependent on prolonged (overnight) stimulation with IL-10 or its analogues according to our studies. On the other hand, blocking of NF-κB p50/p65 by p50/p50 may occur within 30 minutes. We therefore stimulated the cells twice and this could be of importance for an effective suppression of NF-κB binding to DNA.
In this study we compare the effect of IL-10 and its synthetic analogues with respect to their modulary capacity on the binding of NFkappaB to specific motifs, namely the p53 and IL-8 motifs. We find that:

- *IL-10 significantly modulate the binding of NF-kappa to the p53 and the IL-8 motifs.*

- *The C-terminal domain of IL-10, represented by IT9302 appears to be responsible for this effect.*

- *Structural modifications of IT9302 may improve the potency of this IL-10-like effect.*

- *Thus, the new IL-10 analogues, which are structural modifications of IT9302 appears to overcome previous stability problems, which are attributed to IT9302 (data not shown). This enhanced stability could explain the increased potency of the new analogues compared to IL-10 and IT9302.*

8. We further declare that all statements made herein of our own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 10. nov. 2003 Signature: Borbala Gesser

Borbala Gesser

Dated: 10/11-03 Signature:

Christian Grønhøj Larsen

1/12/2002

A Synthetic Peptide Homologous to the Functional Domain of Human Interleukin 10 Downregulates the Expression of the MHC class I Alpha Chain and TAP 1/2 in Human Melanoma Cells.

Running title: An IL-10 peptide homologue inhibits antigen presentation in melanoma.

Mónica Kurte*, Jelena Levitskaya+, Mercedes López*, Alejandro Escobar*, Juan Carlos Aguillón*, Max Petersson+, Jehad Charo#, Rolf Kiessling+ and Flavio Salazar-Onfray*.

(Christian Larsen)

* *Disciplinary Program of Immunology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile.*

+ *Department of Oncology and Pathology, Radiumhemmet, Karolinska Hospital, Stockholm, Sweden.*

Corresponding author:

Dr. Flavio Salazar-Onfray

Disciplinary Program of Immunology, ICBM, Faculty of Medicine, University of Chile.

Av. Independencia 1027, Santiago, Chile. Fax: 56-2-7353346

Email: fsalazar@machi.med.uchile.cl

Key words: IL-10, melanoma, antigen presentation, IT9302, TAP, MHC class I.

Abbreviations used: RT-PCR= Reverse transcriptase polymerization chain reaction; NK=Natural Killer; CTL= cytotoxic T cells; MHC= Major Histocompatibility antigen; HLA= Human leukocyte antigen; PBMC= Peripheral blood mononuclear cells; DC= Dendritic cells; LCL= Lymphoblastoid cell lines; mAb= monoclonal antibody; GM-CSF= Granulocytes and macrophages colony stimulating factor; IFN-γ= Interferon gamma; IL-10=Interleukin-10; LMP=Low molecular protein

Tumor cells treated with IL-10 were shown to have decreased but peptide inducible expression of MHC class I, decreased sensitivity to MHC class I restricted CTL and increased NK sensitivity. These findings could be explained, at least partially, by a down-regulation of TAP1/TAP2 expression. Here IT9302, a nanomeric peptide (AYMTMKIRN), homologous to the C-terminal of the human IL-10 sequence, was demonstrated to mimic these previously described effects of this cytokine on MHC class I related molecules and functions. We observed a dose dependent down-regulation of MHC class I at the cell surface of melanoma cells after 48 hours treatment with IT9302. In line with these results, we also observed a 40 to 50 % inhibition in the expression of both MHC class I alpha chain in peptide treated cells as measured by RT-PCR. A dose dependent inhibition by IT9302 of the IFN-γ mediated induction of MHC class I heavy chain and TAP1 and TAP2 proteins was also found using Western blot. Peptide-treated melanoma cells were shown to be more sensitive to lysis by NK cells in a dose dependent way. Taken together, these results demonstrate that a small synthetic peptide derived from IL-10 can mimic the antigen presentation related effects mediated by the cytokine, such as down-regulation of MHC class I and TAP1 and TAP2 expression, in human melanomas and affect tumor sensitivity to NK cells. The *in vivo* relevance of these effects in tumor immunogenicity are currently being investigated using murine models.

Introduction

IL-10 is a protein of 160 amino acids with a molecular size of 18.5 kD and it exists as a 37 kD homodimer (1). IL-10 was discovered as a T cell growth and differentiation factor (2,3) and has the ability to suppress cytokine production by monocytes (4), activated macrophages (5) and Th1 cells (6). IL-10 can also have pleiotropic immunosuppressive effects, including the capacity to block monocyte dependent T cell proliferation (7), and to inhibit class II MHC expression on monocytes (8), dendritic cells (9) and Langerhans cells (10). IL-10 can also inhibit the upregulation of B7 on monocytes (11), monocyte-associated production of nitric oxides and killing of parasites (12). Over-expression of IL-10 in IL-10 transgenic mice was shown to lead to decreased capacity to reject tumors (13).

Interest in the immune inhibitory effects of IL-10 in relation to anti tumor response was based on the recurrent presence of this cytokine in fresh tumor biopsies or ascites fluid derived from cancer patients (14,15). Expression of IL-10 mRNA was found in various kinds of solid tumors, such as ovarian carcinomas (16), renal carcinomas (17), non-small lung cancer cells (18), and melanomas (19). The preferential expression of IL-10 in metastatic versus primarily lesions of melanomas has been established, indicating an increased metastatic potential of IL-10-secreting melanoma cells (20).

We have previously demonstrated that murine tumor cells treated with recombinant IL-10 (rIL-10) or transfected with the IL-10 gene showed a changed phenotype, characterized by a downregulation in MHC class I expression, resistance to lysis by tumor specific CTL and increased sensitivity to lysis by NK cells related to an inhibition in the function and expression of TAP molecules (21, 22). In human melanoma, we demonstrated that recombinant human IL-10 (rhuIL-10) pre-treated melanoma cells lines became less sensitive to tumor specific CTL and also showed a significant reduction of MHC class I expression (23). These observations have been confirmed in studies where IL-10 was described to downregulate HLA class I and class II and also ICAM-1 molecules in melanoma cell lines (24).

Recently, a synthetic nanomeric peptide IT9302 (Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn), with complete homology to an amino acid sequence located in the C-terminal portion of human IL-10 (residues 152-160) has been shown to be the functional domain of IL-10, sharing several of the functions of this cytokine(25). In this report we have analyzed the effect of this IT 9302 peptide on the expression of MHC class I related antigen presentation proteins in melanoma cells. We demonstrate that the IL-10 homologous peptide downregulates the MHC class I surface expression and also the IFN-γ induced expression of MHC class I heavy chain, TAP1 and TAP2 molecules, affecting NK sensitivity of the treated tumor cells.

Material and methods

Cells lines and peptides

The FM55 melanoma cell line was obtained from the Danish Cancer Society through the courtesy of Dr. J. Zeuthen. The OCM1 is an ocular melanoma line kindly provided by Dr. M. Jager (University of Leiden). These cell lines were maintained in culture in RPMI 1640 medium supplemented with 5% FCS, 1 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate (Life Technologies, Gaithersburg, MD,USA). The nanomeric peptide IT9302 (Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn) (25) was chemically synthesized using an automatic polypeptide synthesizer. The purity of the protein was confirmed to exceed 95 % by HPCL, and the molecular weight was controlled by mass spectroscopy. As a control, an irrelevant nanomeric peptide derived from influenza (HBVc18-27) was used.

Treatment of tumor cells with IT9302 and IFN-γ

Melanoma cells ($6x10^5$ cells in 300 µl) were cultured in 5% RPMI medium in the presence of 1, 10, 100 or 1000 ng/ml of peptide IT9302 or an irrelevant control peptide (100 ng/ml) during 12 to 48 h. To investigate the inhibition of IFN-γ mediated induction of antigen presentation proteins, cells were pre-treated during 2 h with different concentrations of IT9302 peptide or control peptide and then incubated in the presence of only medium, or medium containing 100 or 500 U/ml rIFN-γ IMUKIN(R) (Boehringer Ingelheim, Ingelheim, Germany) for additional 10 h for RT-PCR analysis or 46 h for FACS, or Western blot analysis. A similar approach was used to test the effect of 10 to

100 U/ml of rhIL-10 (sp act:10^7 U/mg; DNAX, Palo Alto, CA. A kind gift of Dr. A. O'garra) on IFN-γ induction of antigen presentation

mAbs and FACS Analysis.

Melanoma cells either untreated or stimulated for 48 with IT9302 and with or without rIFN-γ were washed in PBS and incubated for 30 min on ice in RPMI 5% FBS with mAb W6/32 (anti-MHC class I) (ATCC, Rockville, MD) or mouse IgG as negative control. After two washings, the cells were incubated with a secondary anti-mouse IgG-FITC (DAKO, Hamburg, Germany) for 30 min. Cells were analyzed after four washings. Flow cytometry was performed using a FACScan flow cytometer and analyzed by the Cell Quest analysis program (Becton Dickinson, Heidelberg, Germany).

RNA extraction and RT-PCR assay

Total RNA was extracted from melanoma cells after 12 h. incubation in the presence or absence of IFN-γ, using Chomcznski and Sacchi method. cDNA synthesis was performed by incubating 2 μg of total RNA in DEPC water, oligo-(dT) primer, and reverse transcription buffer (Life Technologies, Gaithersburg, MD,USA) at 65°C for 10 min. and then cooling the mixture immediately to 4°C. To this mixture, 10 Mm DTT, 2.5 mM dNTP's and 200 Units of M-MLV-RT (Life Technologies, Gaithersburg, MD,USA) were added to achieve a final sample volume of 20 μl. To complete the reverse transcription, samples were incubated at 37°C for 1 h. For PCR, 1.6 μg of cDNA was diluted in a buffer solution containing 3 mM MgCl, 0.2-0.5 mM of each primers, 0.4 mM of each dNTP's and 2.5 Units of *Taq* DNA Polymerase (Life Technologies, Gaithersburg, MD,USA) to a final volume of 50 μL and amplified in a thermal cycler (Techne). The PCR primers (Life Technologies, Gaithersburg, MD,USA) used were:5'- CAGGACCAGGTGAACAACAAAGTC-3' and 5'-AAGCCAGTTACTCATCAGGGTGG-3' for TAP-2, and 5'- CCTACGACGGCAAGGATTTACATC-3' and 5'-TCCAGAAGGCACCACCACAG-3' for heavy chain of MHC class I. Amplified bands were analyzed by electrophoresis through a 1.3 % agarose gel and ethidium bromide staining .

Western blot analysis.

Cell pellets from harvested melanoma cell lines ($1*10^6$ cells) were suspended in 100 µl lysis buffer (65 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% mercapto-ethanol, 1% bromophenol blue) and maintained for 15 min at room temperature. Then, samples were sonicated by 10 microns for 30 seconds, warmed at 95°C for 5 min and then centrifuged. Fifteen µl each sample was electrophoresed through a 12 % SDS-polyacrylamide gel. For immunoblots, proteins were electro transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA). Membranes were blocked in phosphate-buffered saline containing 5% milk (low fat) (PBS/milk 5%). All additional Immunostaining steps were performed in phosphate-buffered saline with 3 % milk (PBS/milk 3%) and washed with phosphate-buffered saline 0.05 % Tween20 (PBS-Tween 0.05%). Filters were incubated overnight at 4°C with the corresponding primary antibody; mouse mAb TO-1 anti-TAP1, mouse mAb SY-2 anti-TAP-2 (both kindly provide by Dr. S. Ferrone) or a rabbit polyclonal antibody against MHC class I heavy chain. As an internal control a commercial polyclonal antibody against β-actin was used. After washing with PBS/milk the membranes were incubated with the respective secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse Ig or horseradish peroxidase-conjugated sheep anti-rabbit Ig (Amersham, Buckinghamshire, England) for 2 h at room temperature. Filters were then washed in PBS-Tween 0.05% five times and developed with enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, England).

Cytotoxic assay

LAK cells (lymphokine activated killer cells) were obtained from normal donors PBMC. After a gradient separation with Lymphoprep(R) (Life Technologies, Gaithersburg, MD,USA) $25x10^6$ PBMC were incubated during 5 days in 15 ml RPMI 10 % FBS medium containing 500 U/ml IL-2 (kindly supply by Dr. P.Simon, DuPont Merck Pharmaceutical Co.). A 6 h 51Cr-release assay was used to measure cytotoxic activity of LAK cells against peptide and/or IFN-γ treated melanoma cell lines or K562 cells as positive control. All samples were run in triplicate. Specific lysis was calculated according to the formula: percentage specific lysis = 100 x [(experimental cpm-spontaneous cpm)/(maximum cpm – spontaneous cpm)].

Results

The peptide IT9302, homologous to the functional domain of human IL-10, downregulates the surface MHC class I expression on melanoma cell lines.

The nanomeric peptide IT9302 has been demonstrated to constitute the functional domain of human IL-10 (25). To investigate whether IT9301 could also mimic the capability of IL-10 to downregulate MHC class I surface expression on melanoma cells, we treated the cutaneous melanoma line FM55 and the ocular melanoma line OCM1 with 0, 1, 10 and 100 ng/ml of IT9302 during 48 hours. The peptide concentrations were selected considering that it has been described that 6,125 ng of peptide correspond in molarity to approximately 100 ng/ml of human IL-10 (25). A dose dependent inhibition of MHC class I could be observed in both treated melanomas, with a maximal inhibition of 60 % compared to the controls (Fig. 1). These results were in line with the previously observed effects using rhIL-10 (23). No significantly effect of IT9302 was observed on HLA-A2 or MHC class II expression on the HLA-A2 positive / MHC class II negative FM55 line or on the HLA-A2 negative / MHC class II positive OCM1 melanoma line (data not shown).

The peptide IT9302 and rhIL-10 inhibit the MHC class I induction mediated by IFN-γ

The IT9302 peptide was previously shown to inhibit the IFN-γ induced MHC class II expression on human monocytes (25) in line with the known ability of IL-10 to antagonize the effect that IFN-γ has on antigen presentation (26,27). To investigate if rhIL-10 pretreatment of melanoma cells could inhibit IFN-γ induction of MHC class I, OCM1 melanoma cells were first treated with 0, 10, and 100 U/ml of rIL-10 for 2 h and thereafter with 0, 10 and 100 U/ml of rIFN-γ for an additional 10 h. A seven fold induction of MHC class I heavy chain production by 10 U/ml or 100 U/ml IFN-γ as compared to non treated control cells was observed in Western blot analysis (Fig.2). The IFN-γ induction of heavy chain could be inhibited in a dose dependent manner by pre treatment with rhIL-10, with a maximum inhibition of around 60% compared to non treated cells (Fig. 2).

We next investigated if also the peptide homologue could antagonize the IFN- γ mediated enhancement of MHC class I heavy chain expression. OCM1 melanoma cells were first treated with 0, 1, 10, 100 and 1000 U/ml of IT9302 peptide or 100 U/ml of an irrelevant control peptide for 2 h and thereafter with 100 or 500 U/ml of rIFN-γ for an additional 10 h. In this experiment, a 2-3 fold

Figure 4

IT9302 inhibits IFN-γ mediated induction of TAP-1 and TAP-2 but not LMP-2 in low expressing melanoma cell lines.

A) Melanoma FM55 express low amounts of TAP-2 RNA. Melanoma cells and monocytes, as controls, were treated as described in material and methods and analyzed using RT-PCR. (MW) Molecular weight standard.

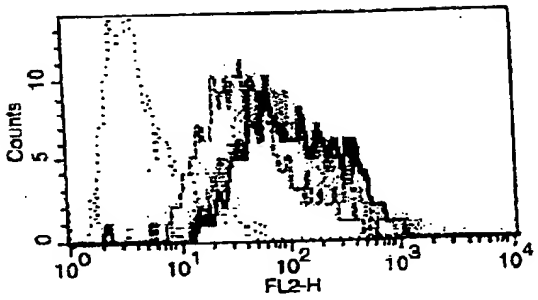
B) Melanoma cell line OCM1 was pre treated with different concentrations of peptide IT9302 or an irrelevant peptide as control and two hours later treated with 100 U/ml of rIFN-γ or with only medium. After 48 hours incubation cell pellets were analyzed by Western blot using TAP1, TAP2, and LMP2 specific antibodies as described in material and methods. β-actin specific mAb was used as internal control.

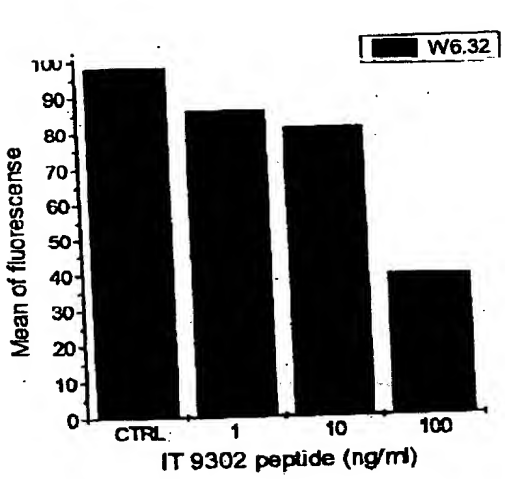
Figure 5

The peptide IT9302 induces a higher sensitivity to LAK mediated cytotoxicity in a melanoma cell line.

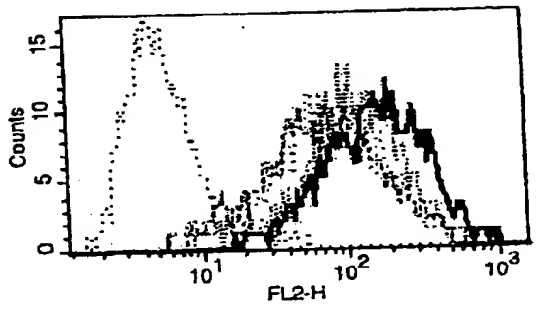
The melanoma cell line FM55 was treated with 1,10,100 ng/ml of peptide IT9302 , with a control irrelevant peptide (100 ng/ml) or with 100 U/ml IFN-γ for 48 hours and then analyzed in a ^{51}Cr release assay against LAK cells as described in material and methods. The NK sensitive cell line K562 was used as positive control.

FM55 melanoma cell line





OCM1 melanoma cell line



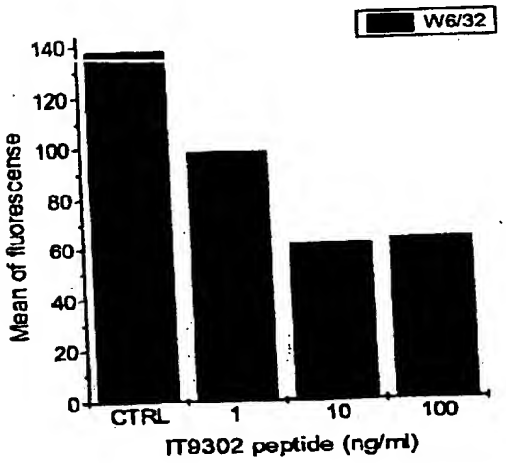
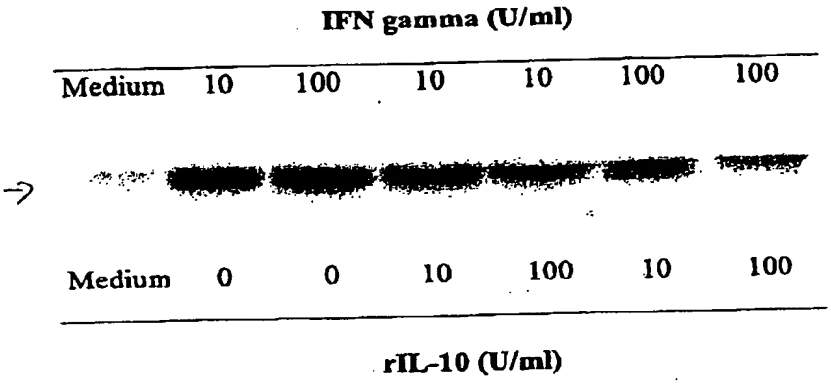


FIG. 1



MHC class I

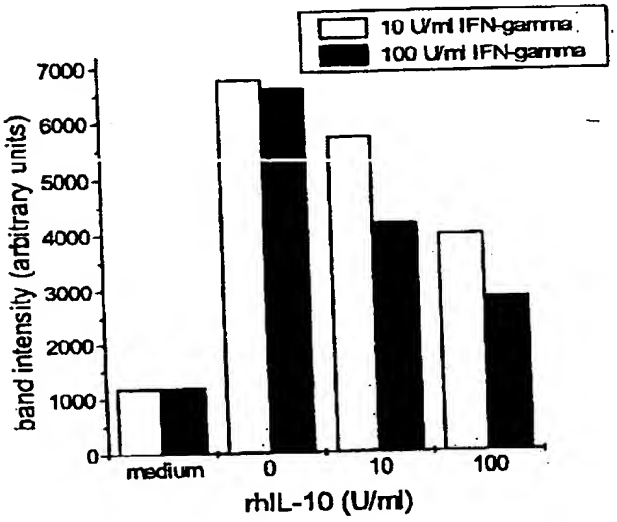
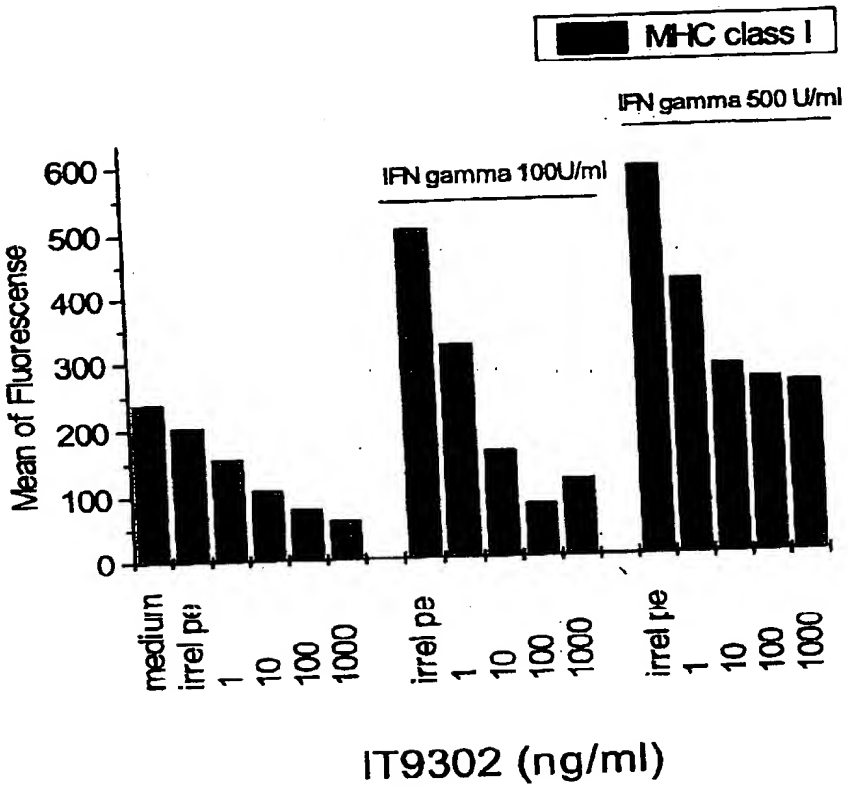


FIG 2

A


MHC class I
IFN gamma 500 U/ml
IFN gamma 100U/ml
Mean of Fluorescense
600
500
400
300
200
100
0
medium
irrel pe
1
10
100
1000
irrel pe
1
10
100
1000
irrel pe
1
10
100
1000
IT9302 (ng/ml)

B

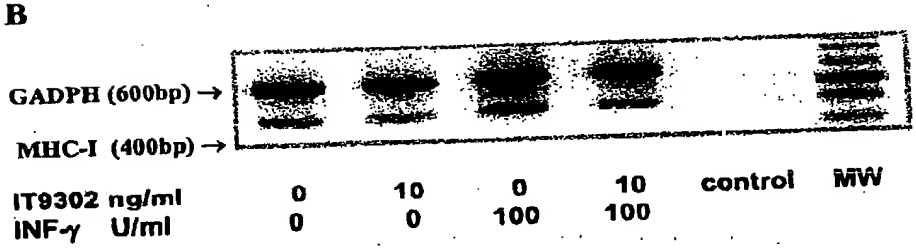

GADPH (600bp) →
MHC-I (400bp) →
IT9302 ng/ml 0 10 0 10 control MW
INF-γ U/ml 0 0 100 100

FIG 3

A


GADPH
TAP-2
FM55 Monocytes MW

B

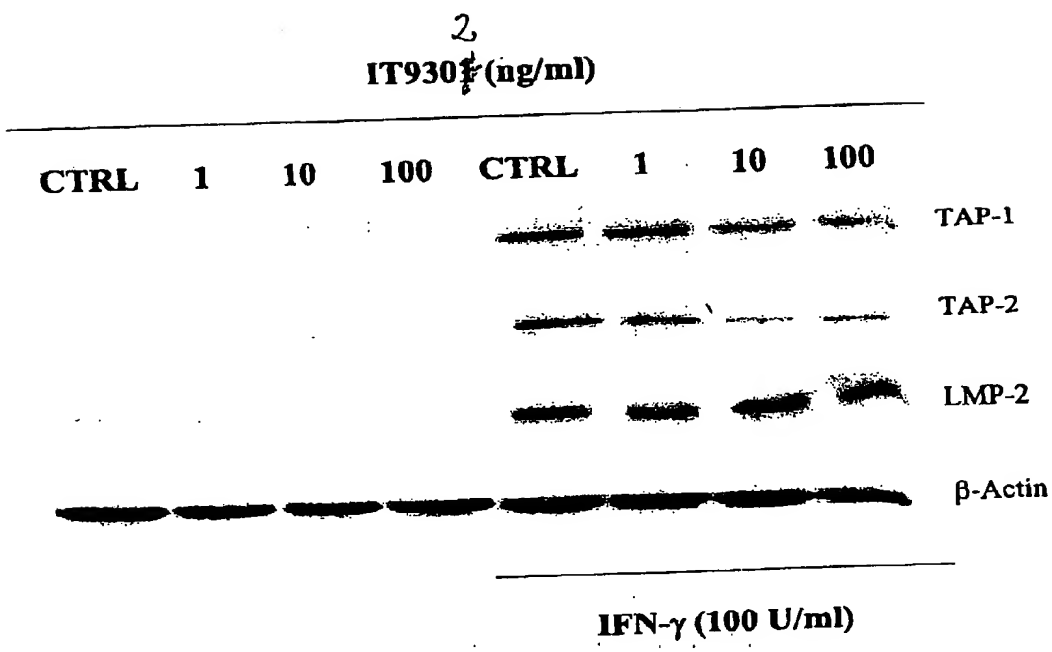

IT930 2 (ng/ml)
CTRL 1 10 100 CTRL 1 10 100
TAP-1
TAP-2
LMP-2
β-Actin
IFN-γ (100 U/ml)

FIG 4

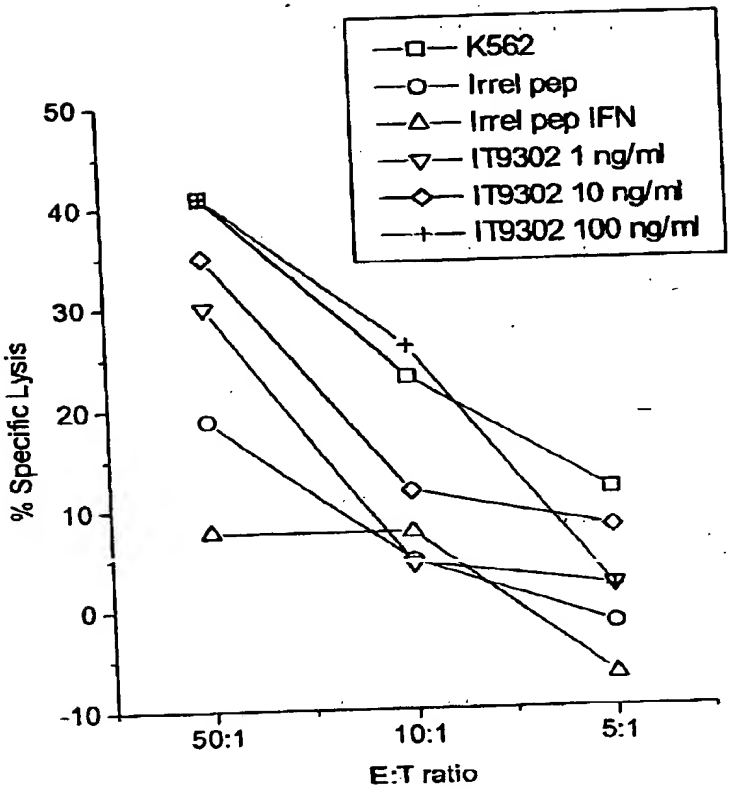

K562
Irrel pep
Irrel pep IFN
IT9302 1 ng/ml
IT9302 10 ng/ml
IT9302 100 ng/ml
% Specific Lysis
50
40
30
20
10
0
-10
50:1
10:1
5:1
E:T ratio

FIG 5

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Activators and target genes of Rel/NF-κB transcription factors

Heike L Pahl*,[1]

[1]Department of Experimental Anesthesiology, University of Freiburg, 79106 Freiburg, Germany

The vertebrate transcription factor NF-κB is induced by over 150 different stimuli. Active NF-κB, in turn, participates in the control of transcription of over 150 target genes. Because a large variety of bacteria and viruses activate NF-κB and because the transcription factor regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules, NF-κB has often been termed a 'central mediator of the human immune response'. This article contains a complete listing of all NF-κB inducers and target genes described to date. The collected data argue that NF-κB functions more generally as a central regulator of stress responses. In addition, NF-κB activation blocks apoptosis in several cell types. Coupling stress responsiveness and anti-apoptotic pathways through the use of a common transcription factor may result in increased cell survival following stress insults.

Keywords: NF-κB; Rel; transcription factors; immune response; stress response; ER overload

NF-κB, a central mediator of the human immune response

The Rel/NF-κB family of eukaryotic transcription factors is comprised of several structurally-related proteins that form homodimers and heterodimers (Chen and Ghosh, 1999, this issue). In vertebrates, this family includes p50/p105, p52/p100, RelA (p65), c-Rel and RelB. These dimers bind to a set of related 10 bp DNA sites, collectively called κB sites, to regulate the expression of many genes. In most cells, Rel/NF-κB transcription complexes are present in a latent, inactive state in the cytoplasm where they are bound to an inhibitor (IκB). As described below, many stimuli can rapidly activate these transcription complexes by freeing them from their inhibitor and enabling them to translocate to the nucleus. The most common Rel/NF-κB dimer in mammals contains p50-RelA and is specifically called NF-κB. For the purposes of this review, NF-κB will be used to refer any induced complex that can be translocated from the cytoplasm to the nucleus and can bind to κB sites.

The transcription factor NF-κB has often been called a 'central mediator of the human immune response'. How was such a reputation established and is it justified? A summary of all stimuli that are known to activate NF-κB (Table 1) and a compilation of its many target genes (Table 2) may provide an answer.

In many cell types, nuclear NF-κB activity is induced by exposure to a wide variety of bacteria or bacterial products (Table 1). Likewise, a host of viruses or their proteins activate NF-κB (Table 1). Bacterial and viral infection certainly present situations where an adequate immune response is vital. That human cells respond to so many different organisms by activating the same transcription factor, NF-κB, is one reason for its reputation as a 'central switch'. Moreover, homozygous disruption in mice of the genes encoding certain members of the Rel/NF-κB family, including those encoding c-Rel, p50 and RelB, leads to defects in the immune response to certain pathogens (Gerondakis *et al*., 1999, this issue).

The active NF-κB transcription factor promotes the expression of over 150 target genes (Table 2). The majority of proteins encoded by NF-κB target genes participate in the host immune response. These include, for example, 27 different cytokines and chemokines, as well as receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across blood vessel walls (Table 2). These target genes alone would merit NF-κB the designation as a 'central mediator of the immune response'.

Many viruses that induce NF-κB activity also harbor NF-κB binding sites in their viral promoters (Table 2). Therefore, it seems likely that a virus would gain a selective advantage from the acquisition of a κB site in its promoter. If the transcription factor is induced either directly through viral infection or indirectly by the ensuing immune response (via inflammatory cytokines, for example), the κB site-containing viral promoter will be transactivated, resulting in enhanced viral transcription. Thus, the organism's own sword is turned against itself. The presence of a κB site in the HIV-1 promoter may have led to the activation of viral replication that was observed during trials in which IL-2 was used to stimulate T-cell replication in HIV-1-infected patients (Kovacs *et al*., 1995). A low level of NF-κB activation is perhaps part of the mechanism by which some viruses, such as EBV, HSV, CMV or HIV-1, maintain their chronic infections.

A compilation of the many pathogens that induce NF-κB and a look at the function of its various target genes certainly validate the reputation this transcription factor has gained as an important regulator of the immune response. Moreover, the fact that viruses often use this protein to their advantage argues that NF-κB activity exerted an evolutionary pressure on these pathogens.

*Correspondence: HL Pahl, Experimentelle Anaesthesie, Klinikum der Albert Ludwigs-Universität Freiburg, Klinik für Tumorbiologie, Breisacher Straße 117, 79106 Freiburg, Germany

Table 1 Inducers of NF-κB activity

Condition	Reference
Bacteria	
EPEC, enterophathogenic *E. coli*	Savkovic *et al.*, 1997
Gardnerella vaginalis	Hashemi *et al.*, 1999
Helicobacter pylori	Münzenmaier *et al.*, 1997
Lactobacilli	Klebanoff *et al.*, 1999
Listeria monocytogenes	Hauf *et al.*, 1994
Micoplasma fermentans	Marie *et al.*, 1999
Mycobacteria tuberculosis	Zhang *et al.*, 1994
Neisseria gonorrhoeae	Naumann *et al.*, 1997
Rickettsia rickettsii	Sporn *et al.*, 1997
Salmonella dublin	Eaves-Pyles *et al.*, 1999
Salmonella typhimurium	Hobbie *et al.*, 1997
Shigella flexneri	Dyer *et al.*, 199
Staphyllococcus aureus	Busam *et al.*, 1992
Bacterial Products	
Diphosphoryl lipid A (*Rhodobacter sphaeroides*)	Lawrence *et al.*, 1995
Exotoxin B	Busam *et al.*, 1992
G(Anh) M Tetra	Dokter *et al.*, 1994
Lipoteichoic acid (*Listeria*)	Hauf *et al.*, 1997
Lipopolysaccharide (LPS)	Sen and Baltimore, 1986a
membrane lipoproteins (*Micoplasma fermentans*)	Garcia *et al.*, 1998; Rawadi *et al.*, 1999
Muramyl Peptides	Schreck *et al.*, 1992
PlcA (Phospholipase) (*Listeria*)	Hauf *et al.*, 1997
PlcB (Phospholipase) (*Listeria*)	Hauf *et al.*, 1997
Staphylococcus enterotoxin A and B (super antigen)	Trede *et al.*, 1993; Busam *et al.*, 1992
Toxic Shock Syndrome Toxin 1	Trede *et al.*, 1993
Viruses	
Adenovirus	Shurman *et al.*, 1989
Cytomegalovirus	Sambucetti *et al.*, 1989
Epstein-Barr Virus (EBV)	Hammarskjöld and Simurda, 1992
Hepatitis B Virus	Siddiqui *et al.*, 1989
Herpes Virus Saimiri	Yao *et al.*, 1995
Human Herpesvirus 6	Ensoli *et al.*, 1989
HIV-1	Bachelerie *et al.*, 1991
Herpex Simplex Virus -1	Gimble *et al.*, 1988
HTLV-I	Leung and Nabel, 1988; Ballard *et al.*, 1988
Influenza Virus	Ronni *et al.*, 1997
Measles Virus	Harcourt *et al.*, 1999
Molony Murine Leukemia Virus	Pak and Faller, 1996
Newcastle disease virus	Ten *et al.*, 1993
Respiratory Syncytial Virus	Mastronarde *et al.*, 1996; Garofalo *et al.*, 1996
Rhinovirus	Zhu *et al.*, 1996a; Zhu *et al.*, 1996b
Sendai paramyxo virus	Hiscott *et al.*, 1989
Sindbis Virus	Lin *et al.*, 1995a
Viral Products	
Adenovirus 5 E1A	Shurman *et al.*, 1989
Adenovirus: E3/19K	Pahl *et al.*, 1996
CMV: ie1	Sambucetti *et al.*, 1989
Double-stranded RNA	Visvanathan and Goodbourn, 1989
EBV: EBNA-2	Scala *et al.*, 1993
EBV: LMP	Hammarskjöld and Simurda, 1992
HBV: HBx	Twu *et al.*, 1989
HBV: LHBs	Hildt *et al.*, 1996
HBV: MHBs^t	Meyer *et al.*, 1992
HCV: Core protein	You *et al.*, 1999
Herpes Saimiri: HVS13	Yao *et al.*, 1995
HIV-1: gp160	Chirmule *et al.*, 1994
HIV-1: Tat	Westendorp *et al.*, 1994
HTLV-I: Tax1	Ballard *et al.*, 1988, Leung and Nabel, 1988
HTLV II: Tax2	Tanaka *et al.*, 1996
Influenza Virus: Hemagglutinin	Pahl and Baeuerle, 1995a
Parvovirus H19, NS1	Moffatt *et al.*, 1996

continued

Table 1 continued

Condition	Reference
Eukaryotic parasite	
Theileria parva	Ivanov *et al.*, 1989
(Inflammatory) Cytokines	
IL-1	Osborn *et al.*, 1989
IL-2	Hazan *et al.*, 1990
IL-12	Grohmann *et al.*, 1998
IL-15	McDonald *et al.*, 1998
IL-17	Shalom-Barak *et al.*, 1998
IL-18	Matsumoto *et al.*, 1997
LIF	Gruss *et al.*, 1992
THANK	Mukhopadhyay *et al.*, 199
TNFα	Osborn *et al.*, 1989; Israël et al., 1989a
TNFβ	Messer *et al.*, 1990
Physiological (Stress) Conditions	
Adhesion	Lin *et al.*, 1995b
Depolarization	Kaltschmidt *et al.*, 1995
Hemorrhage	Shenkar *et al.*, 1996; Shenkar and Abraham, 1997
Hyperglycemia	Yerneni *et al.*, 1999
Hyperosmotic Shock	Courtois *et al.*, 1997
Hyperoxia	Shea *et al.*, 1996
Ischemia (transient, focal)	Gabriel *et al.*, 1999; Li *et al.*, 1999
Liver Regeneration	Tewari *et al.*, 1992; Cressman *et al.*, 1994
Mechanical Ventilation (*in vitro*)	Pugin *et al.*, 1998
Reoxygenation	Rupec and Baeuerle, 1995
Shear Stress	Lan *et al.*, 1994
T-cell Selection	Moore *et al.*, 1995
Physical Stress	
PPME Photosensitization	Legrand-Poels *et al.*, 1995
Ultraviolet irradiation (UV-A, B, C)	Stein *et al.*, 1989
Wounding combined with HeNe irradiation	Haas *et al.*, 1998
γ Radiation	Brach *et al.*, 1991a
Oxidative Stress	
Butyl Peroxide	Munroe *et al.*, 1995
Hydrogen Peroxide	Schreck *et al.*, 1991
Ozone	Haddad *et al.*, 1996
Pervanadate	Imbert *et al.*, 1996
Reoxygenation	Rupec and Baeuerle, 1995
Environmental Hazards	
3,3',4,4'-tetrachlorobiphenyl (PCB77)	Hennig *et al.*, 1999
Chromium	Ye *et al.*, 1995
Cigarette Smoke	Nishikawa *et al.*, 1999
Cobalt	Goebeler *et al.*, 1995
Crocidolite asbesstos fibres	Janssen et al., 1995
Dicamba (herbicide, peroxisome proliferator)	Espandiari *et al.*, 1998
Lead	Ramesh *et al.*, 1999
Nickel	Goebeler *et al.*, 1995
Silica Particles	Chen *et al.*, 1995
Therapeutically used drugs	
1-b-D Arabinofuranosyl-cytosine (ara-C)	Strum *et al.*, 1994
Anthralin	Schmidt *et al.*, 1996
Azidothymidine (AZT)	Kurata, 1994
Camptothecin	Piret and Piette, 1996
Ciprofibrate	Li *et al.*, 1996a
Cisplatin	Nie *et al.*, 1998
Daunomycin	Das and White, 1997; Hellin *et al.*, 1998
Daunorubicin	Wang *et al.*, 1996
Doxorubicin	Das and White, 1997
Etoposide	Bessho *et al.*, 1994
Haloperidol	Post *et al.*, 1998
Methamphetamine	Asanuma and Cadet, 1998
Phenobarbital	Li *et al.*, 1996b
Tamoxifen	Perlini *et al.*, 1999

continued

Table 1 continued

Condition	Reference
Taxol (Paclitaxel)	Hwang and Ding, 1995
Vinblastine	Rosette and Karin, 1995a
Vincristine	Das and White, 1997
Modified Proteins	
Advanced glycated end products (AGEs)	Yan *et al.*, 1994; Wautier *et al.*, 1994
Amyloid Protein Fragment (βA4)	Behl *et al.*, 1994
Maleylated BSA	Misra *et al.*, 1996
Modified (Oxidized)LDL	Rajavashisth *et al.*, 1995; Andalibi *et al.*, 1993
Overexpressed Proteins (ER Overload)	
CFTR	Knorre and Pahl, unpublished observation
Erythropoietin-Receptor	Knorre and Pahl, unpublished observation
[illegible] heavy chain	Pahl and Baeuerle, 1995b
MHC Class 1	Pahl and Baeuerle, 1995b
Receptor Ligands	
Antigen (IgM-Ligand)	Marcuzzi *et al.*, 1989
CD11b/CD18-Ligand (Complement)	Thieblemont *et al.*, 1995
CD28-Ligand (B7-1)	Verweij *et al.*, 1991
CD2-Ligand	Bressler *et al.*, 1991
CD35-Ligand (Complement)	Thieblemont *et al.*, 1995
CD3-Ligand	Tong-Starksen *et al.*, 1989
CD30-Ligand	Berberich *et al.*, 1994
[illegible]-Ligand (gp120)	Chirmule *et al.*, 1994
Fc-2a-Receptor-Ligand (IgG2a)	Muroi *et al.*, 1994
Flt-1-Ligand	Reikerstorfer *et al.*, 1995
Ly6A/E-Ligand	Ivanov *et al.*, 1994
N-CAM	Krushel *et al.*, 1999
Trail-receptor-1-Ligand (Trail)	Schneider *et al.*, 1997
Trail-receptor-2-Ligand (Trail)	Schneider *et al.*, 1997
Trail-receptor-4-Ligand (Trail)	Degli-Esposti *et al.*, 1997
Apoptotic Mediators	
[illegible]-Fas/Apo-1	Rensing-Ehl *et al.*, 1995; Schneider *et al.*, 1997
Mitogens, growth factors and hormones	
Bone morphogenic protein 2	Mohan *et al.*, 1998
Bone morphogenic protein 4	Mohan *et al.*, 1998
Follicle Stimulating Hormone	Delfino and Walker, 1998
Human Growth Hormone	Shen *et al.*, 1997
Insulin	Bertrand *et al.*, 1995
M-CSF	Brach *et al.*, 1991b
Nerve Growth Factor	Wood, 1995; Carter *et al.*, 1996
Platelet-Derived Growth Factor	Olashaw *et al.*, 1992
Serum	Baldwin *et al.*, 1991
TGF-α	Lee *et al.*, 1995
Physiological Mediators	
12(R)-Hydroxyeicosatrienoic acid	Laniado-Schwartzman *et al.*, 1994
Amino acid analogs	Kretz-Remy *et al.*, 1998
Anaphylatoxin C3a	Pan, 1998
Anaphylatoxin C5a	Pan, 1998
Angiotensin II	Li and Brasier, 1996
Basic calcium phosphate crystals	McCarthy *et al.*, 1998
Bradikinin	Pan *et al.*, 1996
C2-Ceramide (N-acetyl-sphingosine)	Andrieu *et al.*, 1995
Cerulein	Gukovsky *et al.*, 1998; Steinle *et al.*, 1999
Collagen lattice	Xu *et al.*, 1998
Collagen Type I	Lee *et al.*, 1995
Des-Arg10-kallidin (B1 receptor agonist)	Schanstra *et al.*, 1998
Double-stranded polynucleotides	Suzuki *et al.*, 1999
f-Met-Leu-Phe	Browning *et al.*, 1997
Heat shock protein 60 (HSP 60)	Kol *et al.*, 1999
Hemoglobin	Simoni *et al.*, 1998
Hyaluronan	Noble *et al.*, 1996
Kainic acid (Kainate)	Kaltschmidt *et al.*, 1995
Leukotriene B4	Brach *et al.*, 1992
L-Glutamate	Guerrini *et al.*, 1995
Lysophosphatidylcholine (LysoPC)	Zhu *et al.*, 1997
PAF (platelet activating factor)	Smith and Shearer, 1994; Mutoh *et al.*, 1994
Potassium	Kaltschmidt *et al.*, 1995
Thrombin	Mari *et al.*, 1994
Chemical Agents	
2-Deoxyglucose	Pahl and Baeuerle, 1995b
Anisomycin	Sen and Baltimore, 1986a
Brefeldin A	Pahl and Baeuerle, 1995b
Calchicine	Rosette and Karin, 1995a
Calcium Ionophores	Novak *et al.*, 1990
Calyculin A	Suzuki *et al.*, 1994
Cobalt chloride	Sultana *et al.*, 1999
Con A	Rattner *et al.*, 1991
Cycloheximide	Sen and Baltimore, 1986a
Cyclopiazonic Acid	Pahl *et al.*, 1996
Forskolin	Delfino and Walker, 1998
Glass fibres	Ye *et al.*, 1999
Linoleic acid	Hennig *et al.*, 1996
L-NMA	Peng *et al.*, 1995
Lysophosphatidic acid	Shahrestanifar *et al.*, 1999
Monensin	Pahl and Baeuerle unpublished observation
N-methyl-D-aspartate	Guerrini *et al.*, 1995
Nocodazol	Rosette and Karin, 1995a
Okadaic Acid	Thevenin *et al.*, 1991
PHA	Sen and Baltimore, 1986b
Phorbol ester	Sen and Baltimore, 1986a
Podophyllotoxin	Rosette and Karin, 1995a
Pyrogallol	Adcock *et al.*, 1994
Quinolinic acid	Qin *et al.*, 1998
Thapsigargin	Pahl *et al.*, 1996
Tunicamycin	Pahl and Baeuerle, 1995b
Vinblastine	Rosette and Karin, 1995a

Where possible, the first publication to report the data is given as a reference

NF-κB, a central regulator of the stress response

NF-κB, however, is involved in the control of transcription of many genes whose functions extend beyond the immediate immune response. For example, NF-κB also regulates the transcription of many acute phase proteins (Table 2). Similarly, there are many activators of NF-κB that are not bacterial and viral pathogens. Therefore, rather than being a central mediator of the immune response, NF-κB perhaps more generally represents a regulator of stress responses. NF-κB activity, for instance, is induced during various physiological stress conditions such as ischemia/reperfusion, liver regeneration and hemorrhagic shock (Table 1). Physical stress in the form of irradiation as well as oxidative stress to cells also induce NF-κB (Table 1). In this context, it appears evolutionarily beneficial that a large variety of stress response genes, such as the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), are in turn activated by NF-κB (Table 2). Again, NF-κB relays the information of an imminent stress and at the same time enacts a response by promoting the transcription of genes whose products alleviate the stress condition.

Besides physiological stress situations, the human body is exposed to environmental hazards and therapeutic drugs, which can also exert a stress. Indeed, NF-κB is activated both by environmental stresses, such as heavy metals or cigarette smoke, and

Table 2 Target genes of NF-κB

Gene	Function	Reference
Cytokines/Chemokines and their modulators		
CINC	Cytokine-induced neutrophil chemoattractant	Blackwell *et al.*, 1994; Ohtsuka *et al.*, 1996
*CXCL 11	Chemokine ligand for CXCR3	Tensen *et al.*, 1999
Eotaxin	β Chemokine, eosinphil-specific	Hein *et al.*, 1997
Gro α-γ	Melanoma growth stimulating activity	Anisowicz *et al.*, 1991
IFN-γ	Interferon	Sica *et al.*, 1992; Sica *et al.*, 1997
IL-1α	Interleukin-1α	Mori and Prager, 1996
IL-1β	Interleukin-1β	Hiscott *et al.*, 1993
IL-1-receptor antagonist	Inhibitor of IL-1 activity	Smith *et al.*, 1994
IL-2	Interleukin-2	Serfling *et al.*, 1989; Hoyos *et al.*, 1989; Lai *et al.*, 1995
IL-6	Interleukin-6, inflammatory cytokine	Libermann and Baltimore, 1990; Shimizu *et al.*,
IL-8	Interleukin-8, α-chemokine	Kunsch and Rosen, 1993
*IL-9	Interleukin-9	Zhu *et al.*, 1996a
IL-11	Interleukin-11	Bitko *et al.*, 1997
IL-12 (p40)	Interleukin-12	Murphy *et al.*, 1995
*IL-15	Interleukin-15	Azimi *et al.*, 1998
β-Interferon	Interferon	Hiscott *et al.*, 1989; Lenardo *et al.*, 1989
IP-10	α Chemokine	Ohmori and Hamilton, 1993
KC	α Chemokine	Ohmori *et al.*, 1995
Lymphotoxin α		Worm *et al.*, 1998
Lymphotoxin β	Anchors TNF to cell surface	Kuprash *et al.*, 1996
MCP-1/JE	Macrophage chemotactic protein, β Chemokine	Ueda *et al.*, 1994
MIP-1α,β	Macrophage inflammatory protein-1, β Chemokine	Grove and Plumbi, 1993; Widmer *et al.*, 1993
MIP-2	Macrophage inflammatory protein-1, β Chemokine	Widmer *et al.*, 1993
RANTES	Regulated upon Activation Normal T lymphocyte Expressed and Secreted, β Chemokine	Moriuchi *et al.*, 1997
TCA3, T-cell activation gene 3	T-cell activation gene 3, β Chemokine	
TNFα	Tumor necrosis factor α	Oh and Metcalfe, 1994
TNFβ	Tumor necrosis factor β	Shakhov *et al.*, 1990; Collart *et al.*, 1990
Immunoreceptors		Paul *et al.*, 1990; Messer *et al.*, 1990
B7.1 (CD80)	Co-stimulation of T cells via CD28 binding	Fong *et al.*, 1996; Zhao *et al.*, 1996
BRL-1	B-cell homing receptor	Wolf *et al.*, 1998
CCR5	Chemokine receptor	Liu *et al.*, 1998
CD48	Antigen of stimulated lymphocytes	Klaman and Thorley-Lawson, 1995
F_c epsilon receptor II (CD23)	Receptor for IgE	Richards and Katz, 1997
IL-2 receptor α-chain	IL-2 receptor subunit	Ballard *et al.*, 1988
Immunoglobulin Cgammal	IgG heavy chain	Lin and Stavnezer, 1996
Immunoglobulin ε heavy chain	IgE heavy chain	Iciek *et al.*, 1997
Immunoglobulin κ light chain	Antibody light chain	Sen and Baltimore, 1986b
Invariant Chain I_i	Antigen presentation	Pessara and Koch, 1990
MHC class I ($I-2K^b$)	Mouse histocompatibility antigen	Israël *et al.*, 1989a; Israël *et al.*, 1989b
MHC Class I HLA-B7	Mouse histocompatibility antigen	Johnson and Pober, 1994
β2 Microglobulin	Binds MHC class I	Israël *et al.*, 1989a; Israël *et al.*, 1989b
T-cell receptor β chain	T-cell receptor subunit	Jamieson *et al.*, 1989
*TNF-Receptor, p75/80	High-affinity TNF receptor	Santee and Owen-Schaub, 1996
Proteins involved in antigen presentation		
Proteasome Subunit LMP2	Subunit of 26S proteasome, cysteine protease	Wright *et al.*, 1995
Peptide Transporter TAP1	Peptide transporter for ER	Wright *et al.*, 1995
Cell adhesion molecules		
ELAM-1	E-selectin, endothelial cell leukocyte adhesion molecule	Whelan *et al.*, 1991
ICAM-1	Intracellular adhesion molecule-1	van de Stolpe *et al.*, 1994
MadCAM-1	Mucosal addressin cell adhesion molecule	Takeuchi and Baichwal, 1995
P-selectin	Platelet adhesion receptor	Pan and McEver, 1995
Tenascin-C	ECM protein controls cell attachment and migration, cell growth	Mettouchi *et al.*, 1997
VCAM-1	Vascular cell adhesion molecule	Iademarco *et al.*, 1992
Acute phase proteins		
Angiotensinogen	Angiotensin precursor, regulates blood pressure	Brasier *et al.*, 1990; Ron *et al.*, 1990
C4b binding protein	Complement binding protein	Moffat and Tack, 1992
Complement factor B	Complement factor	Nonaka and Huang, 1990
Complement Factor C4	Activates extrinsic pathway of complement activation	Yu *et al.*, 1989
C-reactive protein	Pentraxin	Zhang *et al.*, 1995
Lipopolysaccharide binding protein	Binds to LPS receptor (CD14) with LPS	Schumann, 1995
Pentraxin PTX3	Pentraxin	Basile *et al.*, 1997
Serum amyloid A precursor	Serum component	Edbrooke *et al.*, 1991; Li and Liao, 1991
Tissue factor-1	Activates extrinsic pathway of complement activation	Mackman *et al.*, 1991
Urokinase-type Plasminogen activator	Activates fibrinogen for fibrin clot lysis	Novak *et al.*, 1991
Stress response genes		
Angiotensin II	Peptide hormone	Brasier *et al.*, 1990

continued

Table 2 continued

Gene	Function	Reference
COX-2	Cyclooxygenase, prostaglandin endoperoxide synthase	Yamamoto *et al.*, 1995
Ferritin H chain	Iron storage protein	Kwak *et al.*, 1995
*5-Lipoxygenase	Arachidonic acid metabolic enzyme, leukotriene synthesis	Chopra *et al.*, 1992
12-Lipoxygenase	Arachidonic acid metabolic enzyme	Arakawa *et al.*, 1995
inducible NO-Synthase	NO synthesis	Geller *et al.*, 1993
Mn SOD	Superoxide dismutase	Das *et al.*, 1995
NAD(P)H quinone oxidoreductase (DT-diaphorase)	Bioreductive enzyme	Yao and O'Dwyer, 1995
Phospholipase A2	Fatty acid metabolism	Morri *et al.*, 1994
Cell-surface receptors		
A1 adenosine receptor	Pleiotropic physiological effects	Nie *et al.*, 1998
Bradikinin B1-Receptor	Pleiotropic physiological effects	Ni *et al.*, 1998
*CD23	Cell-surface molecule	Tinnell *et al.*, 1998
CD69	Lectin mainly on activated T cells	Lopez-Cabrera *et al.*, 1995
Gal1 Receptor	Galanine receptor, neuroendocrine peptide	Lorimer *et al.*, 1997
[illegible]-1	Receptor for Oxidized low density lipoprotein	Nagase *et al.*, 1998
Mdr1	Multiple drug resistance mediator (P-glycoprotein)	Zhou and Kuo, 1997
Neuropeptide Y Y1-receptor	Pleiotropic physiological effects	Musso *et al.*, 1997
PAF receptor 1	Platelet activator receptor	Mutoh *et al.*, 1994
RAGE- receptor for advanced glycation end products	Receptor for Advanced Glycation End products	Li and Schmidt, 1997
Regulators of apoptosis		
Bfl1/A1	Pro-survival Bcl-2 homologue	Grumont *et al.*, 1999; Zong *et al.*, 1999
Bcl-xL	Pro-survival Bcl-2 homologue	Chen *et al.*, 1999; Lee *et al.*, 1999b
Nr13	Pro-survival Bcl-2 homologue	Lee *et al.*, 1999
CD95 (Fas)	Pro-apoptotic receptor	Chan *et al.*, 1999
FasLigand	Inducer of apoptosis	Matsui *et al.*, 1998
IAPs	Inhibitors of Apoptosis	You *et al.*, 1997; Stehlik *et al.*, 1998
IEX-1L	Immediate early gene	Wu *et al.*, 1998
Growth factors and their modulators		
G-CSF	Granulocyte Colony Stimulating Factor	Nishizawa and Nagata, 1990
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor	Schreck and Baeuerle, 1990
*IGFBP-1	Insulin-like growth factor binding protein-1	Lang *et al.*, 1999
IGFBP-2	insulin-like growth factor binding protein-2	Cazals *et al.*, 1999
M-CSF (CSF-1)	Macrophage Colony Stimulating Factor	Brach *et al.*, 1991b
PDGF B chain	Platelet-Derived Growth Factor	Khachigian *et al.*, 1995
Proenkephalin	Hormone	Rattner *et al.*, 1991
*Thrombospondin	Matrix glycoprotein 1	Adolph *et al.*, 1997
VEGF C	Vascular Endothelial Growth Factor	Chilov *et al.*, 1997
Early response genes		
p22/PRG1	Rat homology of IEX	Schafer *et al.*, 1998*
p62	Non-proteasomal multi-ubiquitin chain binding protein	Vadlamudi and Shin, 1998
Transcription factors		
A20	TNF-inducible zinc finger	Krikos *et al.*, 1992
c-myb	Proto-oncogene	Toth *et al.*, 1995
c-myc	Proto-oncogene	Duyao *et al.*, 1992
c-rel	Proto-oncogene	Hannink and Temin, 1990
IRF-1	Interferon regulatory factor-1	Harada *et al.*, 1994
IRF-2	Interferon regulatory factor-2	Harada *et al.*, 1994
IκBα	Inhibitor of Rel/NF-κB	Haskill *et al.*, 1991; Sun *et al.*, 1993; deMartin *et al.*, 1993
*jun*B	Proto-oncogene	Brown *et al.*, 1995
nfkb2	NF-κB p100 precursor	Lombardi *et al.*, 1995
nfkb1	NF-κB p105 precursor	Ten *et al.*, 1992
p53	Tumor suppressor	Wu and Lozano, 1994
Viruses		
Adenovirus (E3 region)	Adenovirus	Williams *et al.*, 1990
Avian Leukosis Virus	Causes avian leukosis	Bowers *et al.*, 1996
Bovine Leukemia Virus	Causes bovine leukemia	Brooks *et al.*, 1995
CMV	Cytomegalovirus	Sambucetti *et al.*, 1989
EBV (Wp promoter)	Epstein-Barr virus	Sugano *et al.*, 1997
HIV-1	Human immunodeficiency virus	Nabel and Baltimore, 1987; Griffin *et al.*, 1989
HSV	Herpes simplex virus	Rong *et al.*, 1992
Polyoma Virus	Polyoma virus	Ranganathan and Khalili, 1993
Measles virus	Causes measles	Harcourt *et al.*, 1999
SIV	Simian immunodeficiency virus	Bellas *et al.*, 1993
SV 40	Simian virus 40	Kanno *et al.*, 1989
Enzymes		
*Ceramide glycosyl transferase	Glycosphingolipid	Ichikawa *et al.*, 1998
Collagenase 1	Matrix metalloproteinase	Vincenti *et al.*, 1998

continued

Table 2 continued

Gene	Function	Reference
*Dihydrodiol dehydrogenase	Oxidoreductase, oxidation of trans-hydodiols	Ciaccio *et al.*, 1996
*GAD67	Glutamic acid decarboxylase	Szabo *et al.*, 1996
Gelatinase B	Matrix metalloproteinase	He, 1996
GSTP1-1	Glutathione transferase	Xia *et al.*, 1996*
Glucosel-6-phosphate dehydrogenase	Hexose monophosphate	Garcia-Nogales *et al.*, 1999
* HO-1	Hemeoxygenase	Lavrovsky *et al.*, 1994
Hyaluronan synthase	Synthesizes hyaluronic acid	Ohkawa *et al.*, 1999
Lysozyme	Hydrolyzes bacterial cell walls	Phi van, 1996
*PTGIS, prostaglandin synthase	Prostaglandin synthase	Yokoyama *et al.*, 1996
Transglutaminase	Forms isopeptide bonds	Mirza *et al.*, 1997
*Xanthine Oxidase	Oxidative metabolism of purines	Xu *et al.*, 1996
Miscellaneous		
alpha-1 acid glycoprotein	Serum protein	Mejdoubi *et al.*, 1999
Apolipoprotein C III	Apoprotein of HDL	Gruber *et al.*, 1994
*Biglycan	Connective tissue proteoglycan	Ungefroren and Krull, 1996
Cyclin D1	Cell-cycle regulation	Guttridge *et al.*, 1999; Hinz *et al.*, 19
*Cyclin D3	Cell-cycle regulation	Wang *et al.*, 1996b
Factor VIII	Hemostasis	Figueiredo and Brownlee, 1995
Galectin 3	β-galactosidase-binding lectin	Hsu *et al.*, 1996
HMG14	High mobility group 14	Walker and Enrietto, 1996
K3 Keratin	Intermediate filament protein	Wu *et al.*, 1994
Laminin B2 Chain	Basement membrane protein	Richardson *et al.*, 1995
Mts1	Multiple tumor suppressor	Tulchinsky *et al.*, 1997
*Pax8	Paired box gene	Okladnova *et al.*, 1997
*UCP-2	Uncoupling protein-2	Lee *et al.*, 1999a
Vimentin	Intermediate filament protein	Lilienbaum *et al.*, 1990
Wilm s Tumor Supressor Gene	Tumor suppressor	Dehbi *et al.*, 1998
α1-antitrypsin	Protease inhibitor	Ray *et al.*, 1995

Where possible, the first publication to report the data is given as a reference. *Genes contain NF-κB binding sites in their promoter/enhancer regions, but further experiments are required to prove their functionality

by therapeutic drugs, including various chemotherapeutic agents (Table 1). One may speculate that the activators and the target genes of this multifunctional transcription factor have co-evolved. While environmental stresses and xenobiotics activate NF-κB, its target genes include many cell surface receptors, among them the *mdr-1* gene, which encodes the multiple drug resistance mediator. Likewise, while modified proteins such as advanced glycated end products (AGEs) induce NF-κB (Table 1), the AGE receptor (RAGE) is an NF-κB target gene (Table 2).

Another recently recognized cellular stress has been termed the ER-Overload Response (Pahl and Baeuerle, 1997a, 1997b; Pahl, 1999). ER-overload arises from an accumulation of proteins within the endoplasmic reticulum (ER). It can occur under a variety of circumstances:

(1) a sudden increase in the production of proteins which enter the ER, for example during viral infection;
(2) drugs which interfere with ER function thereby leading to protein accumulation in the organelle;
(3) production of mutant proteins, which cannot fold correctly and thus accumulate in the ER; and
(4) an overproduction of wild-type proteins, for example during transient transfection experiments, which overwhelm the ER folding/processing machinery and therefore also accumulate in the organelle (Table 1).

Agents eliciting the ER-Overload Response thus appear under various categories in Table 1.

Cellular stress can result in the most drastic form of cellular self defense, namely programmed cell death or apoptosis. It is now clear that NF-κB can exert both pro- and anti-apoptotic effects in different cells types (Barkett and Gilmore, 1999, this issue). The observation that several stimuli, among them tumor necrosis factor α (TNFα) and binding to the IgM receptor (or cross linking of the receptor with antibodies), can lead both to NF-κB activation (Table 1) and to apoptosis (Laster *et al.*, 1988; Hasbold and Klaus, 1990) suggested that NF-κB induction was pro-apoptotic. Furthermore, both cross-linking of the Fas-receptor by anti-Fas antibodies and binding of the Trail receptors 1 and 2 stimulate NF-κB (Table 1). Moreover, both the Fas-receptor and its ligand are encoded by NF-κB target genes (Table 2). However, cells derived from RelA knockout mice are more susceptible to apoptosis induced by various agents, including TNFα (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996a). Likewise, binding of the Trail receptor-4 induces NF-κB but prevents Trail-mediated apoptosis (Degli-Esposti *et al.*, 1997), and inhibition of NF-κB restores drug-induced apoptosis sensitivity to certain drug-resistant primary leukemic cells and leukemic cell lines (Jeremias *et al.*, 1998). These data, together with the identification of pro-survival *bcl-2* homologs, Bfl1/A1, Bcl-x_L and Nr13, and inhibitors of apoptosis (IAPs) as NF-κB target genes (Table 2), suggest that NF-κB activation is anti-apoptotic in several cell types.

Consistent with a pro-surivival activity for NF-κB, several mitogens and growth factors stimulate NF-κB (Table 1) or are induced by NF-κB (Table 2). Some of these mitogens, such as M-CSF and PDGF, appear to act via an autocrine loop: they activate NF-κB which in turn stimulates transcription of the growth factor

gene. Because mitogens and growth factors stimulate NF-κB activity it is logical that several early response genes are also regulated by this transcription factor (Table 2). Thus, in addition to immune modulation and the more general stress response, NF-κB appears to promote cell survival. Teleologically speaking, it may 'make sense' to couple a stress response factor to anti-apoptotic pathways. This central coordinator evokes an effective response against the stress and ensures that the cell does not succumb in the process.

Many physiological mediators that bear no apparent connection to stress responses also activate NF-κB (Table 1). Among these mediators are several, such as PAF and Bradikinin, that activate NF-κB and whose receptors are NF-κB target genes (Table 2). Perhaps these mediators are released under conditions which have not been recognized or categorized as 'stress'. This, however, is only a question of definition.

Of the many chemical agents that induce NF-κB activity (Table 1), most elicit stress of some sort. Cycloheximide, for example, inhibits protein synthesis, while tunicamycin, brefeldin A, 2-deoxyglucose and monensin disrupt ER function, thereby eliciting ER-overload. Nocodazol, calchicine, podophyllotoxin and taxol interfere with microtubule function. (Rosette and Karin, 1995). Systematic screening of chemical libraries would surely unearth a plethora of additional agents that induce NF-κB, perhaps by interfering with vital cell functions, thereby causing stress.

In addition to the response genes already discussed, NF-κB activation leads to the transcriptional induction of various transcription factor genes, some themselves members of the Rel/NF-κB/IκB family. In this way, NF-κB limits its own activation, in that NF-κB activation results in the new synthesis of its inhibitor IκB (Table 2). Newly-synthesized IκB can enter the nucleus and dislodge active NF-κB from its DNA binding site (Zabel and Baeuerle, 1990, Zabel *et al.*, 1993). Thus, in most cell types, NF-κB activation is transient. However, because NF-κB can induce the transcription of other transcription factors, for instance the proto-oncogene c-*myc* and the tumor suppressor *p53*, an initial NF-κB activation may indirectly induce the transcription of many more genes than the identified 150 targets.

Thus, a more detailed look at inducers and targets of NF-κB suggests that this transcription factor is more than a mediator of the immune response. It appears that NF-κB is activated and induces responses various forms of cell stress and should therefore more generally be termed a 'central mediator of human stress response'. In this context it is interesting to note that certain well-studied stress situations, such as heat shock and the unfolded protein response, do not activate NF-κB. NF-κB activity, therefore, appears reserved for select but widely varied stresses.

New roles for NF-κB?

There are many NF-κB target genes whose properties and function defy classification (listed under Enzymes and Miscellaneous in Table 2). Several of these target genes, such as vimentin, laminin, collagenase and gelatinase, appear to involve NF-κB in the regulation of cell structure and micro-environment. Such an adaptation response may also serve to reduce cell stress, however, more data are required to evaluate a role for NF-κB in these processes. Likewise, the observations that NF-κB regulates transcription of the cyclin D1 gene (Guttridge *et al.*, 1999; Hinz *et al.*, 1999) and may also participate in cyclin D3 gene transcription (Wang *et al.*, 1996b) are very intriguing. If NF-κB controls cyclin transcription, this would implicate NF-κB in cell-cycle progression. However, more data are required to substantiate this idea.

Specificity of the NF-κB response

NF-κB participates in the transcription of over 150 target genes. Are all activated when NF-κB is induced? How can this transcription factor maintain any selectivity or specificity?

For NF-κB activation the selectivity resides mainly in the cell type targeted. Not all cell types respond equally to a given stimulus, either because they lack the cognate receptor or because they lack the required signal transduction molecules (discussed in Karin, 1999, this issue). Thus, not every stimulus listed in Table 1 will activate NF-κB in every cell type examined.

Several different mechanisms confer selectivity on the transcriptional response to NF-κB activation. These include:

(1) the combinatorial response of promoter/enhancer regions, and
(2) The selective activation and binding of individual Rel/NF-κB proteins.

The combinatorial response

The promoter enhancer regions of most genes contain more than one transcription factor response element. Therefore, more than one transcription factor is usually required to induce effective transcription of a given gene. For the target genes listed in Table 2, NF-κB activity is necessary for efficient transcription. Thus, mutation of the κB site abrogates transcription of these promoters. However, NF-κB may not be sufficient for full transcription, as other transcription factors are also required. This combinatorial regulation of transcription provides specificity to a given response. While a given stimulus may activate NF-κB, if it fails to activate additional transcription factors, the target gene will not be transcribed fully.

We have demonstrated such selectivity using stably transfected cell lines, which express a chimeric p50/VP16 protein. In this fusion protein, the p50 DNA-binding domain confers specific binding to κB sites, while the HSV VP16 transactivation domain provides potent transcriptional activation. Expression of the p50/VP16 protein was placed under the control of a tetracycline repressable promoter in CMS-5 fibroblast cells. While induction of p50/VP16 was sufficient to activate transcription of the GM-CSF gene in these cells, transcription of the NF-κB target genes IL-1, IL-2 and IL-6 was not induced (Meerpohl and Pahl, unpublished observations). Thus, despite the almost overwhelming number of NF-κB target genes, the individual gene is selectively activated under specific circumstances.

Selective activation of Rel/NF-κB family members

The various members of the Rel/NF-κB family differ in their preference for specific DNA-binding sites (Kunsch *et al.*, 1992). Using random oligonucleotides and a DNA-binding assay, Kunsch *et al.* (1992) selected optimal DNA-binding sites for p50, RelA and c-Rel, as homodimers. The preferred binding site differs for each homodimer. Not surprisingly, such specificity is also seen *in vivo*. For example, several NF-κB target genes have been reported to contain binding sites that preferentially bound by RelA homodimers (e.g., ICAM-1 (Ledebur and Parks, 1995) and IL-4 (Casolaro *et al.*, 1995)).

Thus, the availability of different Rel/NF-κB hetero- and homodimers, whose synthesis and activation may be controlled by distinct signal transduction pathways, provides an additional level of selectivity in NF-κB-mediated gene transcription.

Demonstration of NF-κB activation and discovery of target genes

The simplest and most reliable assay to demonstrate NF-κB activation is the electrophoretic mobility shift assay (EMSA, (Müller *et al.*, 1997)). When combined with the appropriate controls, i.e. unstimulated control cells, as well as competition and supershift assays, the EMSA is both sensitive and accurate. Transient transfection experiments using a κB site-driven reporter gene are also frequently used. The appropriate controls include either a reporter gene lacking the κB sites, or, even better, a reporter gene preceded by mutated κB sites. Inhibition of reporter gene expression by co-transfection of an expression vector for the NF-κB inhibitor IκB provides an additional confirmation of specificity. All of the inducers listed in Table 1 have been demonstrated to activate NF-κB using one of these assays.

What makes a gene an NF-κB target gene? Not all genes listed in Table 2 are *bona fide* NF-κB target genes. That is, genes in Table 2 preceded by an asterisk merely contain a putative NF-κB binding site in their promoter/enhancer region. To establish that a gene is truly regulated by NF-κB, the following experiments must be performed:

(1) NF-κB binding to the putative DNA site must be shown in an EMSA, preferably using cell extracts from a tissue which usually expresses the gene under investigation, and

(2) the promoter/enhancer region must be cloned in front of a reporter gene and functional importance of the κB site must be demonstrated by mutagenesis.

These two experiments are required before a gene can be considered an NF-κB target. However, these data can be misleading if, for example, NF-κB can bind to a DNA sequence, but this site or adjacent sequences are occupied by other proteins *in vivo*. In such a scenario, NF-κB would bind the isolated site *in vitro* in an EMSA. Mutation of this site would result in a loss of function in reporter gene assays, but only because binding of the adjacent unidentified protein is lost. Therefore, for final proof of NF-κB involvement, *in vivo* DNA footprinting of the κB site should be performed. This, however, has not been done for the majority of the target genes listed in Table 2. Nevertheless, the vast majority of these genes are expected to be true NF-κB target genes. Moreover, it is likely that cDNA microarray technologies and genomic sequencing will identify many other NF-κB target genes.

Summary

This article lists and categorizes the known inducers and target genes of the pleiotropic transcription factor NF-κB known to date. Compilation of these data reveals that the vast majority of NF-κB inducing agents or conditions represent a form of stress to cells. In response, many NF-κB target genes function to alleviate cell stress. In addition, NF-κB has recently been shown to inhibit apoptosis in several cell types. Therefore, NF-κB may act as a central integrator of stress responses and cell survival pathways. The rapid rate at which new NF-κB inducers and target genes are being identified suggests that this transcription factor may coordinate additional cellular functions.

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